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EVALUATION OF TISSUE REACTION WITH USAGE OF PIGMENTED NANO-FILLED SILICONE ELASTOMER (A-2000)

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ABSTRACT: Maxillofacial prostheses, such as silicone elastomers, are crucial for reconstructing congenital or acquired defects to help patients lead normal lives. However, long-term tissue reactions to these materials remain debated despite their effectiveness.
OBJECTIVES: This study aimed to assess the tissue reaction on intrinsically pigmented nano-filled platinum based silicone elastomer A-2000.
METHODOLOGY: 54 intrinsically pigmented biomedical silicone elastomers (A-2000) samples were evaluated for cell reaction, where the Human Gingival Fibroblasts were subjected to cell viability in which cell lines were isolated from human gingival keratinized tissue and then seeded in wells, to achieve the evidence of cell viability in response to the cured silicone elastomer (A-2000) when silicone is intrinsically pigmented through MTT assay indirectly assess the silicone samples to see their effect on cell viability using one-way ANOVA.
Results A significant differences among all study groups at all-time intervals ($p = 0.001$). At 24 hours, the RG group differed significantly from both the control and TG groups ($p < 0.001$), and the control group differed from the TG group ($p = 0.003$). At 48 hours, significant differences were found between the control group and both the RG ($p = 0.020$) and TG groups ($p = 0.004$). At 72 hours, a significant difference was observed only between the control and RG groups ($p = 0.001$).
CONCLUSION: Decreased cell viability was consistently observed throughout the incubation intervals, with statistically significant differences detected within an acceptable range.
KEYWORDS: Maxillofacial defects, platinum Silicone Elastomer, Intrinsic Pigmentation

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INTRODUCTION

Maxillofacial prosthesis is used to rehabilitate maxillofacial defects either acquired or congenital, although it is considered as a psychological issue among the majority of patients around the world (1). History mentions that old Egyptians and Chinese used wax and resins to rehabilitate missing portions of the head and neck region(2).

In 1946 Silicones were introduced by Barnhart for extra-oral prosthesis, and then became one of the most promising maxillofacial materials(3). New advances are being made to overcome their weaknesses they became more popular over other materials as they have a range of good physical properties (such as excellent tear and tensile strength) over a range of temperatures, are easier to manipulate, chemical inertness, low degree of toxicity, high degree of thermal and oxidative stability and durability(4, 5).

Furthermore, they can be stained intrinsically and/or extrinsically to give a more natural appearance and when adequately cured, silicone elastomer resist absorbing organic materials that lead to bacterial growth(6)

In 2000, Factor II (Lakeside, AZ) introduced A-2000 as the first generation of a 1:1 mixture of platinum-catalyzed silicone, which is a room-temperature vulcanizing (RTV) material.

Function and esthetics are the main requirements for patients who seek maxillofacial prosthetic treatment, but in terms of ideal (chemical, physical, and mechanical) qualities of maxillofacial material(7), it should be biocompatible with human tissues that do not cause irritation or initiate inflammatory or foreign reaction in the body, and certainly should not be carcinogenic(8, 9). According to various studies, there is no absolute inert material; a biological response takes place when a material is in contact with living tissue(5). This depends on the host, material, and conditions that affect the material according to its function. Where this response is a dynamic ongoing process that continues over time(10). The maxillofacial prosthesis generally lasts from 13 to 28 months and needs frequent replacement (11-13). It is important to find out the biological and toxicological properties of dental materials according to their clinical usage(14).

Various material cytotoxicity in vitro tests mentioned in the research, such as the methyl-thiazolyl-tetrazolium assay (MTT), which is

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considered a standard colorimetric assay used to assess the metabolic activity of cells, it applies to most cell types(15). Generally, studies pertaining biocompatibility of platinum silicone elastomer (A-2000) are scarce. It is therefore necessary to understand it especially when this material is subjected to aging and pigmentation.

The study aimed to assess the tissue reaction on intrinsically pigmented nano-filled platinum-based silicone elastomers A-2000. The null hypothesis was that there was no statistically significant difference in using platinum-filled silicone elastomer material (A-2000) whether intrinsically pigmented or non-pigmented.

MATERIALS AND METHODS

Preparation of samples

To standardize the testing procedure a stainless steel mold fabricated according to standards determined by the International Organization for Standardization (ISO) for vulcanized rubber testing, in which each Silicone elastomer specimen is a disc shape of dimension 10 mm in diameter and 2 mm in thickness(16, 17).

Fabrication of samples

A-2000 platinum-based cured silicone elastomer (Factor II Inc. Lakeside, AZ, USA) consisting of a base (Part A) and a catalyst (Part B) was mixed in a ratio of 1:1 by weight, this mixture was done by the manufacturer's instructions The vulcanization process recommended being 25°C with overnight cure according to manufacturer (18), for pigmented silicone equal weight of 1:10 of intrinsically pigmented ground pigments (Factor II Inc. Lakeside, AZ, USA) was incorporated into the mixture before polymerization. After polymerization, the specimens were carefully removed from the molds and the flash was trimmed away.

The sample size was estimated by assuming a 5% alpha error and 80% study power. The mean (SD) cell survival rate after 72 hours in the pigmented elastomers group was 1.72 (0.19), (19) while it was 0.37 (0.10) in the non-pigmented elastomers and 0.49 (0.05) in the plain silicone elastomer group (control). Software, Sample size was based on Rosner's method (20) calculated by Gpower 3.0.10(21).

The silicone specimens (N= 54) have been divided into three groups as follows: Reference group (RG) (n=27) A-2000 platinum silicone elastomer (Non-pigmented); Test group (TG) (intrinsically pigmented) (n=27)

Before microbiological evaluation, the polymerized silicone elastomer specimens were sterilized in a vacuum autoclave for 15 minutes at 121 °C to prevent bacterial contamination(8, 18)

Isolation and culture of gingival fibroblasts

Attached keratinized gingival tissues were processed for isolation and characterization of human gingival fibroblasts (HGFs). A signed informed consent was obtained from the donors according to a protocol approved by the Ethical Committee of the Faculty of Dentistry, Alexandria University, Egypt.

For HGF isolation, attached keratinized gingival tissues have been obtained from healthy patients undergoing clinical crown lengthening.

Gingival tissues were washed and fragmented into small fragments with dimension (1x1mm) and cultured in tissue culture dishes in low glucose Dulbecco's modified Eagle's medium (LG-DMEM) supplemented with 10% fetal bovine serum, 2 mm L-glutamine, 1% penicillin/streptomycin and left in a humidified incubator with 5%Co2 at 37C. The growth media changed every 2-3 days for 15 days to permit the growth of the tissue-explanted fibroblasts. Immunophenotyping characterization of cultured fibroblasts using the flow cytometer. Immunofluorescence on cells was analyzed using Becton Dickinson, FACS caliber flow cytometer equipped with Cell Quest software(22).

Cell experimental treatment

Experiments are designed to assess the indirect effects of silicone after 24, 48, and 72 hrs in growth media (GM) on cellular viability among three time intervals 24, 48, and 72 hrs. To assess the in-direct cellular response to the chemical leachate from each silicone, samples will be placed in six-well plates that will contain culture media. Growth complete media and conditioned media collected through the three-time intervals from pigmented and non-pigmented aged silicone samples will be transferred to the cells and incubated for 24, 48, and 72 hrs.; after which the MTT viability test have been done(18).

Cell viability assessment (MTT Assay)(18, 19)

HGF cell viability was measured using the MTT test; the cells were seeded in three 96-well plates (Trevigen, HelgermanCT, Gaithersburg, MD, USA.) with a density of 5×10^3cells/well and incubated 24 hrs. for attachment. Cells were exposed to different conditions; 96-well plates wells divided into three groups; Control group (CG) where cells cultured in complete growth media, Reference Group (RG) where cells cultured in conditioned media of plain platinum-based silicone elastomer in which this group divided furthermore to 3sub-groups (RG24), (RG48) and (RG72) according to time intervals media collected after24, 48 and 72 hours respectively. After 24 hours the media of 96 well plates were changed by conditioned media of (RG) and (TG) and fresh complete media, and so on for 48 and 72 hrs.

Fibroblasts were grown as monolayer cultures in the culture media mentioned above. Once confluent, cells were trypsinized and plated into

two 96-well plates with a uniform seeding density of 7_103 cells/well and incubated with different formulations for 24 h. After 48 h the media from the corresponding plates were aspirated, replaced by MTT solution, and incubated for 4 h. Lastly, the formazan blue crystals were dissolved in DMSO and the absorbance at 570nm was measured by ELISA well-plate reader (Tecan, Infinite F50, M€annedorf, Switzerland). The values obtained were compared with the control that was regarded as 100% living cells(23)

MTT reagent in DMEM (100 μL/well) and incubated for 3-4 hrs. The formed crystals were solubilized in solubilization solution Dimethyl Sulfoxide (DMSO) and placed on a plate shaker in the dark for 15 minutes [35], next the optical density was read at 570 nm and 630 nm as a reference wavelength using a UV spectrophotometer. The survival rates of the blank control group which involved untreated cell cultures set to represent 100% proliferation.

Statistical Analysis

Mean and standard deviation were used for data presentation. One Way ANOVA followed by Tukey's post hoc test with Bonferroni correction was employed for analysis. Cell viability was calculated as (optical density of test group ÷ optical density of cellular control group) × 100. The significance level was set at $p < 0.05$ and analyzed using IBM SPSS version 23.

RESULTS

Tables 1 and 2 represent the cell viability at different time intervals among the study groups. The cell viability percentage of study group material was evaluated relative to the periods of 24, 48, and 72; compared with the control (cultured in complete growth media). Throughout time intervals, there was a decrease in the cell viability of all groups.

After 24 hours, all study group samples showed lower cell viability with a statistically significant difference between groups with a p-value of <0.0001, While there was a decrease in cell viability over time in the same group which is significant with p p-value of <0.0001. After 48 hours the cells showed the least viability among RG (92.53 %) with a statistically significance value with a p-value of 0.204**.** The optical density of the gingival cells among the study group was evaluated relative to periods of 24, 48, and 72 hours. According to one-way ANOVA, there was a significant difference between all the study groups in all time intervals with a P-value of 0.001, Where the significant difference was found at 24 hours between RG and (control, TG) with a P-value <0.001 while between control and TG (P= 0.003). After 48 hours the significant values were found when comparing the OD (Optical density) of the control to RG and TG 0.020 and 0.004 respectively, while after 72 hours the significant difference values were only found when the control group compared to RG (P =0.001)

As shown in Table 2, After 24 hours of aging it was found that RG showed significantly higher optical density compared to a control group with P-value <0.001 and near 0.8 and 0.76 respectively. Throughout time intervals there was a significant decrease in (OD) of the two study groups RG and TG (P-value <0.001), For RG a significant difference was found upon comparing OD after 24 and (48 and 72) hrs. with P value of 0.001. and significant value was found to be (P= 0.004) between 48 hrs. and 72 hrs.

The cell viability percentage of study groups was evaluated relative to the periods of 24, 48, and 72 hours; compared with the control group. Throughout time intervals there was a significant decrease in cell viability of all groups but with favorable cell viability of more than 90% for RG and TG for time intervals of 24 hours and with slight cytotoxicity.

In study groups RG and TG with P values of <0.001 and 0.019 respectively, According to one-way ANOVA the significant value was only found to be between RG and TG after 24 hours with a P value of < 0.001. According to RG the significant difference between time intervals was found when comparing 24 hrs. to 48hrs and 72hrs with a P value of < 0.001, while for TG the significant difference was found only to be between 24hrs and 72hrs with p-value = 0.022.

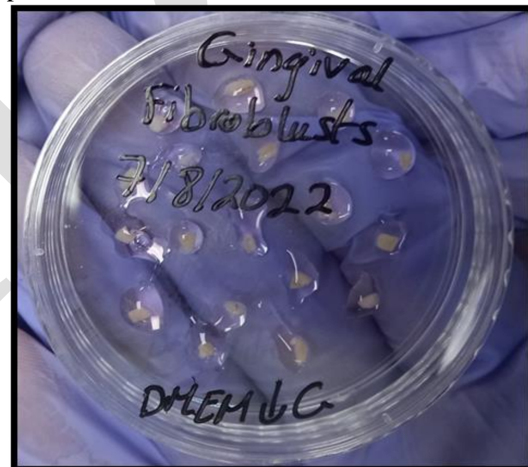

Figure 1: culturing gingival tissue fragments in tissue culture dishes.

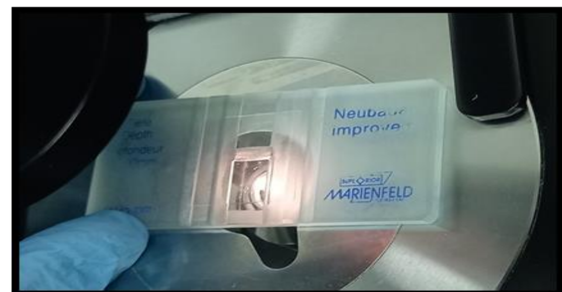

Figure 2: confirming cell detachment under microscope.

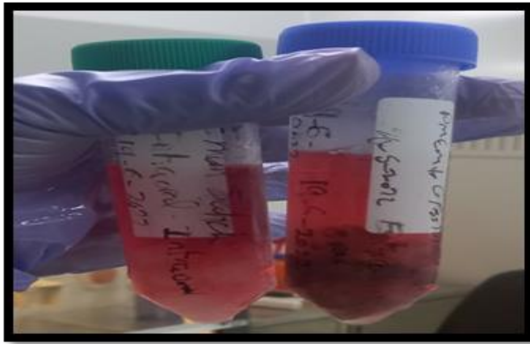

Figure 3: collection of conditioned media of silicone samples and storing them till cytotoxicity test.asured by MTT test.

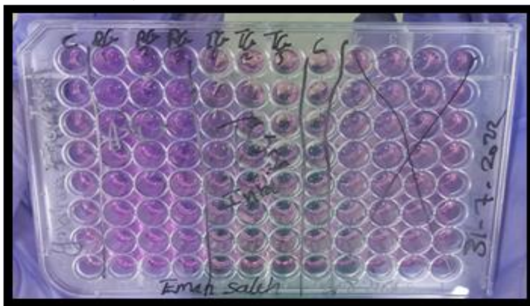

Figure 4: The cell viability of Human gingival fibroblasts me

Table 1: Comparison of optical density for gingival tissues among the study groups at different time intervals

Time intervals	Control (n=9)	RG (n=9)	TG (n=9)	*P* value
	Mean (SD)			
24 hours	0.80 (0.03)	0.86 (0.02)	0.76 (0.02)	<0.0001*
48 hours	0.77 (0.04)	0.72 (0.04)	0.68 (0.05)	<0.0001*
72 hours	0.84 (0.04)	0.77 (0.03)	0.73 (0.04)	<0.0001*
***P* value**	0.003*	<0.0001*	<0.0001*	
Pairwise	P_1=0.236, P_2=0.097, P_3=0.002*	P_1<0.0001*, P_2<0.0001*, P_3=0.004*	P_1<0.0001*, P_2=0.237, P_3=0.043*	

*Statistically significant at p value<0.05. P_1: comparison between 24 hours and 48 hours, P_2: comparison between 24 hours and 72 hours, P_3: comparison between 48 hours and 72 hours.

Table 2: Pairwise comparison between groups regarding optical density for gingival tissues at different time intervals

Time intervals	Groups	Compared to	P value
24 hours	Control	RG	<0.0001*
		TG	0.003*
	RG	TG	<0.0001*
48 hours	Control	RG	0.020*
		TG	<0.0001*
	RG	TG	0.239
72 hours	Control	RG	<0.0001*
		TG	<0.0001*
	RG	TG	0.115

*Statistically significant at p value<0.05

DISCUSSION

Facial defects involving both intraoral and extraoral areas, known as combined defects, require specialized treatment. Prosthodontists must integrate their artistic and clinical skills to create prostheses that are both functional and aesthetically pleasing. Anaplastologists must carefully choose biocompatible maxillofacial materials that are safe for use in the body (24). The effectiveness and biocompatibility of these materials for prosthetic rehabilitation after surgery are still uncertain. This study aimed to evaluate the impact of intrinsically pigmented RTV silicone elastomer on the cell viability of human gingival fibroblasts, using the RTV Platinum silicone elastomer material (A-2000).

The material was selected based on its widespread use among respondents, with RTV platinum-catalyzed silicone elastomers from Factor II (Lakeside, AZ) being particularly common. Among these, the Factor II silicones (A-2186, A-2186F, A-2000) were the top three choices. However, another study found that the physical and mechanical properties of A-2186 might degrade after exposure to seven environmental variables: natural weathering, normal aging, two types of adhesives, two types of cleaning agents, and cosmetics(25). Continued development of extraoral maxillofacial materials is necessary to meet the needs of professionals designing and fabricating prosthetics. Current investigations focus on the physical and mechanical properties, toxicity, color stability, and longevity of commonly used materials. Cosmesil intrinsic skin pigments, suspended in silicone fluids for enhanced color stability and dispersion, were used to replicate both normal and racial skin tones(26). The amount of pigment used in maxillofacial prosthetic elastomers can affect the material's structure and properties. This study investigated the effect of pigmented Cosmesil series M511 on tissue cell viability using cell culture methods(27). It addresses concerns that pigment levels might alter material structures, particularly in facial prosthetics, which interact with living human skin over extended periods. Such interactions, including perspiration and sebum absorption, may affect prosthetic durability. Although the types of silicone elastomers used in this study differed from those used by El-Fray et al., the findings were consistent with their results(28).

The cytotoxicity of silicone elastomers for maxillofacial applications was assessed using an MTT assay. This method, commonly used to evaluate dental materials, measures live cells by detecting the conversion of yellow MTT salt to purple MTT-formazan, catalyzed by mitochondrial succinate dehydrogenase(29). The blue formazan accumulates within cells, and after cell membrane lysis with isopropanol, the solubilized formazan can be quantified using a spectrophotometer(18, 23). Although previous research reported that L-929 mouse fibroblasts are more sensitive than primary

human gingival fibroblasts, The A-2000 material demonstrated high cell viability when tested indirectly on L-929 mouse fibroblasts. Enhancing A-2000 elastomers with titanium oxide nanoparticles or fumed silica did not affect the biocompatibility profile of the mouse fibroblasts, even over time(8).Therefore limitation of this study is evaluation of biocompatibility of silicone after being subjected to aging circumstances

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