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Comparison of *in vitro* biocompatibility of silicone and polymethyl methacrylate during the curing phase of polymerization

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Abstract: Adverse events have been reported with acrylic bone cements. However, current test standards for acrylic materials fail to characterize the potentially harmful monomers released during the curing stage. In clinical applications, materials are implanted into the human body during this phase. Silicone may be a safer alternative to acrylic cements. Silicone is used in medical applications for its biocompatibility and stability characteristics. Previously, no study has been completed which compares silicone to acrylic cements. In this study, both materials were injected into the cell medium during the curing process which more accurately reflects clinical use of material. Initially, cell cultures followed ASTM standard F813-07 which fails to capture the effects of monomer released during curing. Subsequently, a modified cell culture method was employed which evaluated cytotoxicity while the materials cured. The objective of this study was to capture toxicity data during curing phase. Thus, the test method employed measured and excluded the impact of the exothermic reaction temperature of polymethyl methacrylate (PMMA) on cell growth. The concentration of PMMA monomer was measured at 1 and 24 h after injecting PMMA into culture plates in a manner consistent with established cell growth methodologies. Our results indicate current *in vitro* cytotoxicity assays recommended by ASTM standards are unable to reveal the real cytotoxic effect caused by methyl methacrylate monomers during polymerization. Our modified experiment can more accurately illustrate the true nature of the toxicity of materials and improve assay results. In these tests, silicone based elastomeric polymers showed excellent cytocompatibility. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2018.

Key Words: biocompatibility/hard tissue, bone cement–PMMA, acrylic, cell–material interactions, cytotoxicity, silicone elastomeric polymer

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INTRODUCTION

Polymethyl methacrylate ("PMMA") bone cement ("acrylic bone cement") has been used in orthopedic surgery for over 60 years. While PMMA is widely available for use in arthroplastic procedures of the hip, knee, and other joints for the fixation of polymer or metallic prosthetic implants to living bone, it has many shortcomings.[1–3] The use of PMMA has expanded in recent years to include percutaneous vertebral augmentation. Interestingly, the standard test methods used to evaluate the safety and efficacy of PMMA have not caught-up to the commercial uses of PMMA. The standard biocompatibility testing recommended by US Food and Drug Administration (FDA), and established by ISO 10993 and ASTM F748,[4,5] which designs for testing cured materials, may result in misleading conclusions for vertebroplasty or kyphoplasty procedures. The toxicity during the curing phase of PMMA polymerization has been overlooked.[6,7] While fully polymerized PMMA is considered as nontoxic, monomer methyl methacrylate (MMA) can take up to 24 h to fully polymerize, during which time the MMA can be released leading to toxicity problems. The mixed cement is inserted in the dough state when used for joint replacement, however, the injection process for percutaneous vertebral argumentation requires the cement to be more liquid, thus less polymerized and more toxic MMA monomer can be released. Studies have shown that after 1 min of

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incubation monocytes, granulocytes and endothelial cells disintegrate when exposed to partially polymerized PMMA.[8] Moreover, polymerization of PMMA produces heat which is known to cause necrosis in surrounding bones.[9] Even though the study of Toksvig-Larsen et al. in 1991 where they measured the heat generated during polymerization in total joint replacements claimed the heat generated during cement polymerization was removed by the flowing blood in the surrounding bone, their study was performed on joint replacement where there is a thin layer of cement and a large bone surface.[10] However, there is a bolus of cement with a lower surface area to volume ratio in vertebraplasty, which is of course a factor needs to be considered. Testing of cured materials fails to characterize these potentially harmful issues of PMMA. Thus, regulators and researchers have erroneously concluded that PMMA is nontoxic based on the results generated from standard biocompatibility tests that only apply to cured materials.[11]

Numerous adverse issues have been reported with acrylic bone cements including loosening or displacement of the prosthesis, bone cement implantation syndrome, adverse tissue reaction, and among others.[12,13] One of the major drawbacks of bone cement in joint replacement is cement fragmentation and foreign body reaction to wear debris, resulting in prosthetic loosening and periprosthetic osteolysis. The production of wear particles from roughened metallic surfaces and from the PMMA cement promotes local inflammatory activity, resulting in chronic complications to hip replacements.[14] Histologically, a layer of synovial like cells which line the bone cement interface supported by a stroma containing macrophages and wear particles, has been described in loose prostheses.[15] A third of dense fibrous tissue contains PMMA, polyethylene and metallic debris. Activated macrophages express cytokines including interleukin-1, interleukin-6, and tumor necrosis factor alpha, which mediate periprosthetic osteolysis.[14,16–18] It is neither osteoinductive nor osteoconductive and does not remodel.

Given the issues with PMMA, alternative materials have been developed for vertebral augmentation procedures. A safer potential alternative is silicone, which is used in many applications because of the biocompatibility and stability characteristics.[19] Silicone's chemical stability and elastic nature with lower brittleness are beneficial for clinical use involving long-term implantation.[20] The silicone elastomer usually consists of two material components and a dispensing system which blends the two components during injection into the injured site of a fracture. The material cures *in situ* to form a stable, nonresorbable elastomeric polymer. Once the desired viscosity has occurred, the material in injected into the affected area and the mixture will polymerize *in situ* in approximately 8–15 min. The mixture generates no heat during polymerization.

This study was developed to compare the cytocompatibility of silicone elastomer with PMMA during the curing stage. Both materials are used in percutaneous vertebral augmentation and cure *in situ*. To simulate clinical use, both materials were injected into the cell medium during the curing process. Cell cultures and treatments were initially followed by ASTM standard F813-07, which is specified for cell direct contact evaluation from ASTM standard F748, and subsequently performed by a modified cell culture method to better evaluate the cytotoxicity resulted from material curing stage. The aim of the study was to evaluate the monomer toxicity released from PMMA simulated to clinical applications.

MATERIALS AND METHODS

All chemicals used for preparation and analysis were of analytical grade and obtained from Sigma-Aldrich if not stated otherwise. Simplex P cement was from Stryker (Mahwah, NJ). Murine MC3T3-E1 preosteoblast cell line was from ATCC (Manassas, VA). Alpha-modified minimum essential medium (α-MEM) was purchased from Invitrogen (Carlsbad, CA). The silicone elastomer system (VK 100) was provided by BONWRx (Lansing, MI). The VK100 System consists of a cartridge containing the two VK100 material components and a dispensing system which blends the two components during injection into the injured site of a vertebral compression fracture. The material cures *in situ* to form a stable, nonresorbable silicone polymer. VK100 material is comprised of two flowable paste components that are mixed prior to application into a flowable, viscous premix that can be injected into the desired structure to augment the tissue in that area. Each component is contained in side by side, separate cylinders in the prefilled, sealed cartridge. The two components are mixed in a 1:1 ratio with a sterile static mix element that is attached to the output of the cartridge with a bayonet fitting.

Cell culture

Pregrown murine MC3T3-E1 preosteoblast cells (ATCC) were used for the cell culture. Briefly, MC3T3-E1 cells were seeded at a density of 1.5×10^4 cells/well (six-well plate). MC3T3 cells were cultured in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 10 m*M* β-glycerophosphate (Sigma), and a 1% (v/v) antibiotic mixture of penicillin and streptomycin at 37°C in a humidified incubator with 5% CO_2.

Cytotoxicity assay followed ASTM standard

We utilized the ASTM standard F813-07 using eluent saturated filter article to contact with cells to evaluate the cytotoxicity of PMMA and silicone elastomer eluent. Briefly, 2.5 mL of material was loaded into a 5 mL syringe and injected into 3 mL of sterile 0.9% saline in a six well plate. After incubating material at 37°C in saline for 24 h the saline was transferred to another well containing sterile filter article (4 cm^2) and incubated for an additional 24 h. Finally, the soaked filter article was placed directly on top of a semiconfluent monolayer of preosteoblast (MC3T3-E1) cells and cultured for 24 h before removing filter article and analyzing deoxyribose nucleic acid (DNA) content, released lactose dehydrogenase (LDH) and staining with Live/Dead stain. Cell toxicity was determined by measuring the release of LDH from dead or dying cells into the culture medium by

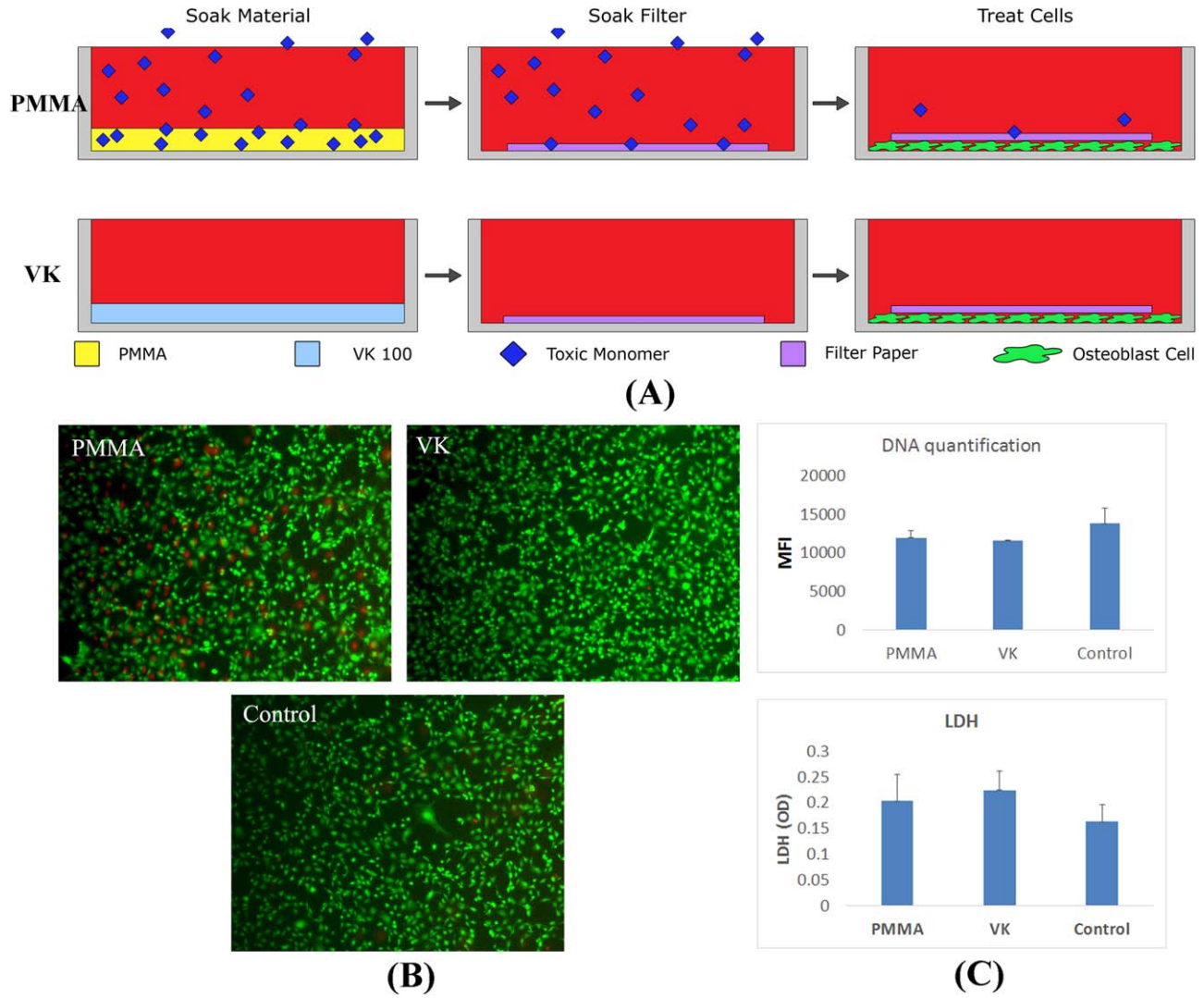

FIGURE 1. Live/dead stained cells after 24 h growth in direct contact with material extract soaked filter article (A). Living cells are stained green whereas cells with damaged membrane are stained red (B). Total DNA content measured using fluorescent assay to determine cell proliferation ($n = 3$) and assay of cytotoxicity performed by measuring LDH content in cell medium (C) ($n = 4$).

colorimetric method following the manufacturer's instruction (Roche Diagnostics BmbH, Indianapolis, IN).[21] Blank culture medium was used as a blank control, and the total cell lysate was used as a positive control. LDH activity was expressed as absorbance (optical density) per mg protein. Live/Dead staining was performed using the Live/Dead® viability/cytotoxicity staining kit (Invitrogen, Camarillo, CA).[22] Stained cells were observed under excitation/emission peaks at 495/517 nm (fluorescein isothiocyanate (FITC), green) and 650/670 nm (Cy5, red) and digital images were acquired using a Zeiss light microscope (US ZEISS, Brighton, MI) equipped with a Toshiba CCD, and these images were analyzed using the Image Pro image analysis software package (Media Cybernetics, Silver Spring, MD).

Modified cytotoxicity assay

Instead of using eluent saturated filter article to contact with cells, the PMMA and eluent were cocultured with cells in the wells. In the second experiment, we directly injected 0.1 mL of the materials to the cell culture wells using a 1 mL syringe. Since both PMMA and silicone elastomer are lighter and can float on the medium surface without contacting with cells, we expect all monomer release during PMMA and VK setting to directly affect the cells [shown in Figure 2(A)]. Cells were cultured for 24 h in the presence of material before evaluating DNA content and performing Live/Dead staining, as described above.

Cell proliferation

Total DNA content was determined using the Quant-iT PicoGreen assay in which a fluorescent product is generated when double stranded DNA complexes with the PicoGreen reagent. Following 7 days of culture the three- and two-dimensional nanofiber (NF) scaffolds were washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells before lysing (150 μL/dish) with cell lysis reagent (CelLytic MT Cell Lysis Reagent, Sigma-Aldrich, St. Louis, MO). Following manufacturer protocol, the assay

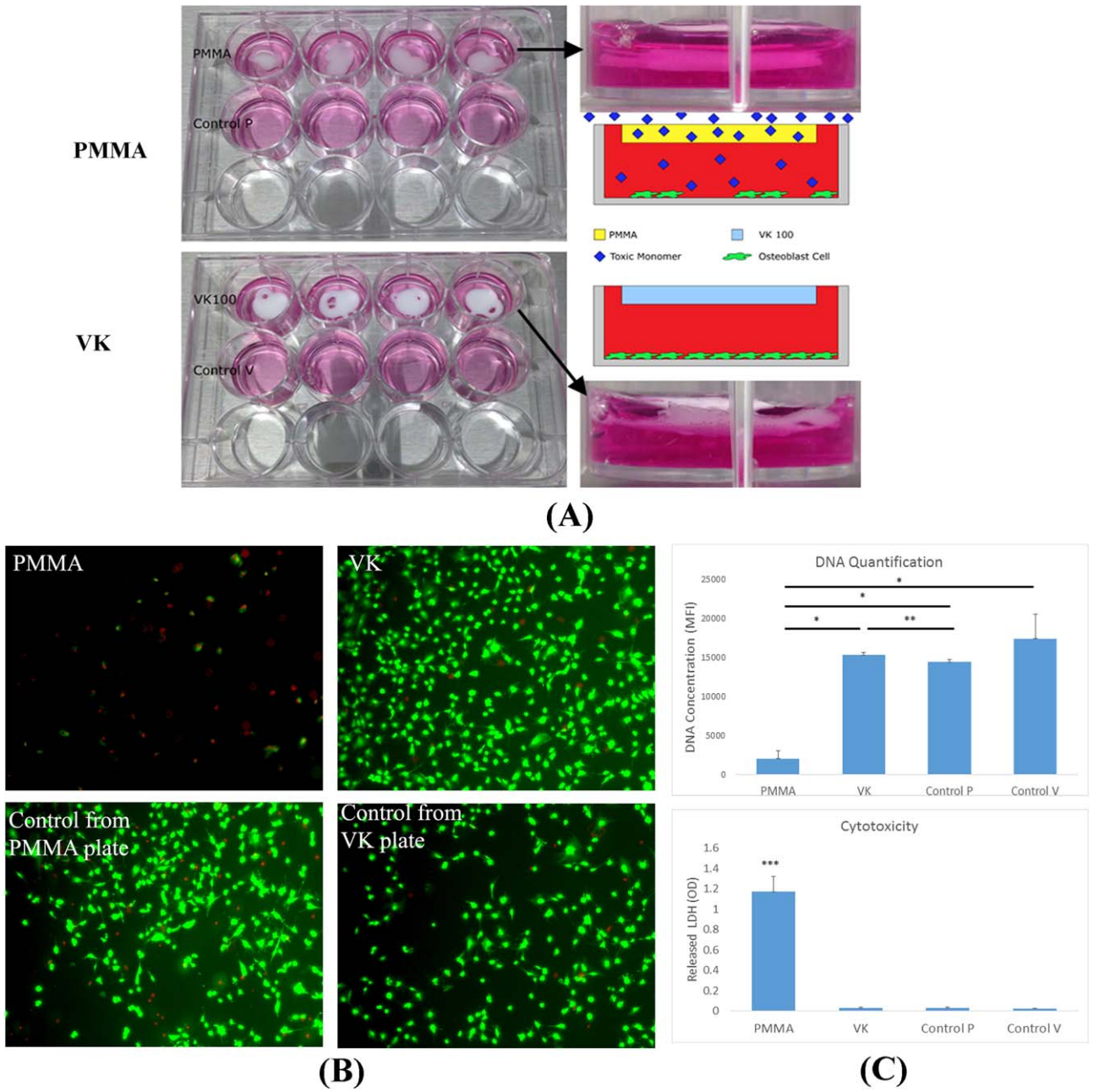

FIGURE 2. A: Images taken of plate layout showing material floating on cell medium and corresponding control wells. Side view of individual well with material floating demonstrating no contact with well bottom (right). Additionally, diagram of side view demonstrating the release/dissolution of toxic monomer into medium and location of cells in well (right). B: Live/dead stained cells after 24 h grown in the presence of PMMA or VK material and the corresponding controls. Living cells are stained green whereas cells with damaged membrane are stained red. C: Total DNA content measured using fluorescent assay to determine cell proliferation (top). Statistically significant differences indicated using ($*p < 0.05$; $**p < 0.01$) ($n = 6$). Measure of cytotoxicity performed by measuring LDH content in cell medium (bottom) Statistical differences from all other groups indicated by ($***p < 0.001$) ($n = 8$).

working solution was prepared through a 20-fold dilution of the provided Tris-ethylenediaminetetraacetic acid (EDTA) buffer with DNase-free distilled water and combined with the PicoGreen reagent in a ratio of 200:1, respectively. Cell lysate was then combined with equal parts PicoGreen working solution and following a brief room temperature incubation, fluorescence values measured at 528 nm after excitation with 485 nm light. In addition to cell lysate, samples of known DNA concentration were analyzed to accurately determine DNA content.

Exothermic reaction temperature and monomer release assay

The exothermic reaction temperature measurement was conducted to exclude the impact from heat generated during PMMA setting on cell growth. Within 1 min after doughing

time, gently pack approximately 25 g of the dough into the mold manufactured from ASTM standard F451-08. A digital thermometer was placed to the medium with as-injected PMMA and VK dough to record the temperature change. The concentration of PMMA monomer was measured at 1 and 24 h after injecting PMMA into culture plates in a manner similar to the cell experiments. Absorbance spectrum of PMMA monomer was obtained using an ultraviolet-visible spectrophotometer (BioTek Synergy HT). Briefly, 100 μL of test medium (10 mg/mL) were placed in a 96-well ultraviolet plate and mounted onto the spectrophotometer. A scanning wavelength range of 200–350 nm and a step size of 1 nm were set. Distilled water was used as the liquid as opposed to culture medium to allow the optical density of the solution to be measured without interference at 300 nm. A standard curve of monomer concentration ranging from 0.0 to 18.8 mg/mL was used to determine actual monomer concentration in test solutions.

Data processing and analysis

Quantitative data were presented as mean ± standard deviation, and an analysis of variance followed by the Least Significant Difference (LSD) *post hoc* assessment was applied to compare the groups using Statistics 18.0 software (SPSS Inc., Chicago). Differences were considered significant if their p values were <0.05 ($/p < 0.05$) and highly significant if their p values were <0.01 ($//p < 0.01$).

RESULTS

In accordance with direct contact method recommended by ASTM standard F813-07, an eluent saturated filter article specimen was placed on the cell layer in the culturing well. MC3T3-E1 cells in close contact with the filter article show normal proliferation among groups with mostly live (green) cells and minor dead (red) cells in staining images [Figure 1(B)]. Notably, PMMA group shows obviously more dead cells (red) compared to the other groups. However, quantitative results of total DNA amount and released LDH amount from dead cells [Figure 1(C)] show no significant difference ($p > 0.05$) in cytotoxicity between PMMA and other groups. The result should indicate the eluent extracted from both PMMA and silicone elastomer was not harmful for cells. The contradiction from qualitative and quantitative results raised a hypothesis that something extracted from curing PMMA to filter article could still kill some cells but cannot showing in quantification results. Thus, a modified cell culture experiment was performed to confirm the hypothesis.

In the modified cell culture experiment, MC3T3-E1 cells challenged with monomer released during PMMA curing stage showed evidence of cellular apoptosis as visualized under Live/dead staining image. Not only total cell numbers are significantly less but also are more dead cells (red) in comparison to any other group. We found significant less ($p < 0.05$) total DNA from cells in PMMA group than other groups. Moreover, the significantly higher release of LDH ($p < 0.001$) as the evidence of cellular apoptosis is also

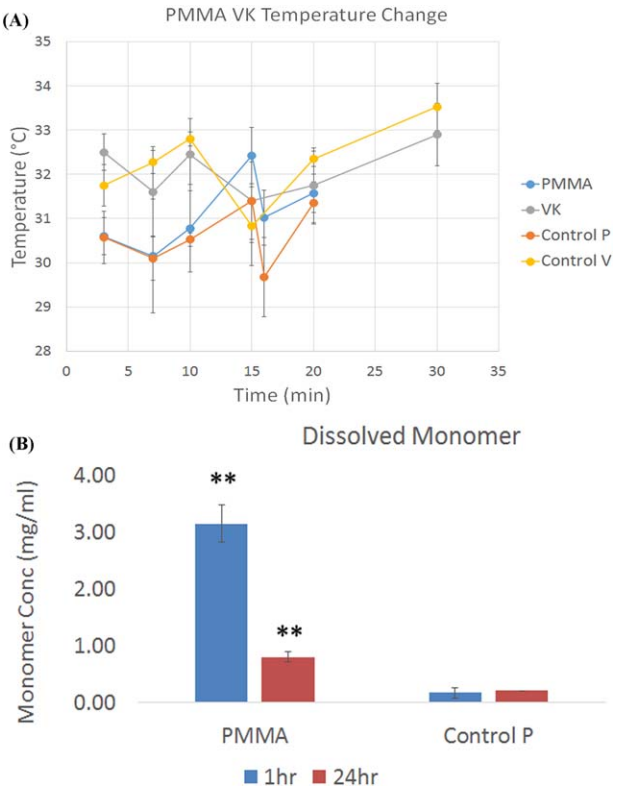

FIGURE 3. A: Recorded temperature of cell medium following injection of PMMA and the corresponding controls. Before and after injection plate was warmed in a 37°C water bath. B: Concentration of dissolved monomer in cell medium at 1 and 24 h following injection of material into cell culture. Statistical differences from PMMA at 1 and 24 h indicated by (**$p < 0.01$ against all other groups; **$p < 0.01$, and ***$p < 0.001$ against silicone elastomer at 1 h; ##$p < 0.01$ against all control groups).

confirmed [Figure 3(C)]. Both qualitative and quantitative results from this modified cell culture experiment indicate the monomer released from PMMA *in situ* during curing stage had great cytotoxicity to cells, while such critical information was missed by following the ASTM F813-07 in previous experimental setup. Notably, we noticed the control group in PMMA plate (control P) shows more cellular apoptosis compared to control group in silicone elastomer plate (control V) [Figure 2(A)] from live/dead staining and DNA quantification, which means the monomer released from PMMA passed through wells to the neighboring control-group wells and sacrificed cells. Even though no statistical difference ($p > 0.05$), control V shows better cell viability from DNA quantification [Figure 2(C)]. Cells treated by silicone elastomer shows equivalent proliferation rate to cells in control V and even better than control P, which confirms the superior cytocompatibility of silicone elastomer over PMMA.

During initial setting stage, the exothermic reaction also releases tremendous heat causing tissue necrosis. In modified cell culture experiment, we directly injected PMMA cement into cell culturing medium and the cellular apoptosis resulted from the heat cannot be ruled out. Therefore, a measurement of exothermic reaction temperature for both

silicone elastomer and PMMA was performed. We found the culture medium stayed at around 30–33°C during the setting period of injected PMMA and silicone elastomer for about 30 mins [Figure 3(A)]. No obvious exothermic peak and deviation from controls was observed, which ruled out the possibility that cells be sacrificed by heat generated from the amount of materials injected to each well. Hence, as we proposed throughout this study, the "overlooked monomer" could play a critical role of killing cells during PMMA setting within the first 24 h after injection. Thus, the monomer concentration in each well once injected was measured at 1 and 24 h time points [Figure 3(B)]. The concentration of monomer in cell medium was 3.2 ± 0.3 mg/mL in PMMA containing groups 1 h after injecting material, statistically higher than control group ($^{**}p < 0.01$). The initial concentration of monomer leads to lethal damage for cell viability, and most monomer is evaporated however with residue after 24 h still negatively interrupt cell growth. This result explained the reason why saturated filter article followed by ASTM F813-07 showed much less cytotoxicity due to the "escaped" monomer.

DISCUSSION

PMMA was first synthesized by Dr. Otto Rohm in 1901, and later manufactured for applications in aircraft production. The first PMMA bone cement use in orthopedics is widely credited to John Charnley and Dennis Smith, who in 1958, used it for total hip arthroplasty.[23] This was a significant milestone in the advancement of Orthopedic surgical procedures. However, PMMA was not subjected to a complete review by FDA. The material was "grandfathered." On May 28 1976,[24] the US passed the Medical Device Amendments to the Federal Food, Drug, and Cosmetic Act ("Act"). The Act expanded the jurisdiction of the FDA authority to include medical device regulation. The expansion of the FDA's regulatory authority was controversial and had many legal hurdles.[25] To ease the adoption of the statute, medical devices that were on the market before 1976 (i.e., preamendment devices) were automatically cleared for sale in the USA or grandfathered. PMMA was used for use in hip and knee prosthetic fixation as well as dental applications, prior to 1976 and was classified as a "Preamendment Device" under the Act.[26] As a result, PMMA bone cement was not subjected to the stringent FDA requirements for clearance, and was automatically allowed for commercial distribution in the USA.[26]

When PMMA is applied as bone cement, the liquid monomer polymerizes around the prepolymerized powder particles to form hardened material.[27] Once the liquid and powder components are mixed during the routine application of bone cement in a surgical procedure, the polymerization process is divided into four phases: mixing, waiting, working, and hardening.[28] The material becomes chemically stable once the polymerization completes. However, unreacted or leaching MMA monomer has been reported over decades for its extreme toxicity.[2,3] Experimental and clinical studies have documented that monomers may cause a wide range of adverse health effects such as irritation to skin, eyes, and mucous membranes, allergic dermatitis, stomatitis, asthma, neuropathy, disturbances of the central nervous system, liver toxicity, and fertility disturbances.[3] Despite of all these clinical complications, PMMA bone cement products are still dominant as biopolymers in orthopedic applications.

Current biocompatibility evaluation standards of PMMA recommended by FDA as class II device (ISO 10993 or ASTM F748) all specified the *in vitro* cytotoxicity assays.[4,5] However, all standards are focusing on the evaluation of postcuring or polymerized material, which represents a "safe region" for PMMA since most unreacted monomer has been leaching out and the polymerized content are physiochemical stable. In both ISO 10993 and ASTM F748 standards, direct contact of cytotoxicity assays is recommended to use a vehicle that allows for extraction of constitutes from tested sample soaking medium to coculture with cells. Since most toxic monomers have been leaching out and unable to extract from vehicle, the subsequent cell culturing results are unconvincing. Our first cytotoxicity study shown in Figure 1 is following the methodology described in ISO 10993 and ASTM F748 standards. Obviously, PMMA seems "safe" to cells for the extracted monomers from polymerized sample. However, in our modified experiment, PMMA was directly tested during polymerization by inject the mixing material to cell culture wells and result shows significant cytotoxicity (Figure 2), which demonstrated current standards specified for bone cement application are inadequate to evaluate the monomer toxicity of PMMA especially during polymerization.

VK 100, a silicone elastomer product, was developed as an alternative for PMMA. Silicone has been used in many applications because of their stability, low surface tension, and lack of toxicity. The first published report of silicone elastomers being implanted in humans was in April 1946, when Dr. Frank H. Lahey told of his use of these materials for bile duct repair. Citing its elastic properties, he reported, "It is flexible, it will stretch, it will bounce like rubber and it can be cast in any shape" (Lahey, 1946).[20,29] A recent clinical study with 82 patients involved shows silicone elastomer showed good results in terms of Oswestry disability index and visual analog score improvement for patients treated with balloon kyphoplasty performed with silicone elastomer over a follow-up period of 12 months.[30] In our *in vitro* cell culturing study, VK 100 show excellent cytocompatiblity in both first experiment followed by standards and second modified experiment, which demonstrated its superiority over PMMA in safety during polymerization.

The limitations of this study are: (1) the measurement of released monomer needs to be further improved for higher sensitivity and accuracy; (2) only osteoblastic cell line was conducted in this study to evaluated monomer cytotoxicity. Immuno-response and osteoclastic induction from released monomer need further exploration.

In the future, a Fourier-transform infrared spectroscopy (FTIR)/Raman spectrometry will be utilized to quantify the released monomer from PMMA polymerization and a time

versus concentration dependence study will be performed. The direct contact cytotoxicity and tissue response from released monomer will be further evaluated *in vivo* through a mouse air-pouch model.

CONCLUSION

In this study, both an *in vitro* cytotoxicity experiment followed by ISO and ASTM standards and a modified cell culture experiment were conducted to evaluate the cytocompatibility of PMMA and VK 100. Our results indicate current *in vitro* cytotoxicity assays recommended by those standards are unable to reveal the real cytotoxic effect caused by MMA monomers during polymerization, while our modified experiment showed the hidden result. The release of MMA during-curing stage of PMMA shows potential threat to biocompatibility, which cannot be directly monitored from current standards from using fully cured specimen. In our study, VK 100, a silicone product, showed excellent cytocompatibility in experiments testing both cured and during-curing specimen, which makes it a promising alternative for PMMA in future orthopedic applications.

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