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Investigation of DNA damage in cells exposed to poly (lactic-co-glycolic acid) microspheres

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Abstract: Poly (lactic-co-glycolic acid) (PLGA)-based materials are widely investigated for drug delivery and tissue engineering applications. Despite their popularity the genotoxic potential of PLGA has not been investigated. In this study, the comet assay, a sensitive assay for DNA damage, was used to evaluate potential genotoxicity in model cell types exposed to PLGA microspheres. Human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSCs) cells were exposed to PLGA microspheres (0.4–6 mg/mL) and DNA damage assessed at 24 h, 4 days, and 7 days. DNA damage was not identified after 24 h. However, after 4 and 7 days of exposure to 2 and 6 mg/mL of PLGA microspheres a significant elevation of DNA damage in both cell types was observed. The PLGA microspheres did not exhibit any cytotoxic effects on the cells under the conditions tested. Our results suggest that PLGA may have a genotoxic effect on cells. A broader investigation of the PLGA genotoxic profile in biological systems is needed. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 284–291, 2017.

Key Words: biomaterials, genotoxicity, HUVECs, MSCs, tissue engineering

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INTRODUCTION

Poly (lactic-co-glycolic acid) (PLGA) is one of the most popular polymers under investigation for medical applications due to its biodegradability and biocompatibility.[1] Microspheres of PLGA are in clinical use as drug delivery systems for cancer therapy[2–4] and under extensive research for many clinical needs in tissue engineering and drug delivery. PLGA is a copolymer, which has been prepared in methods that allow for sustained delivery of both hydrophobic (e.g., small molecule drugs) and hydrophilic (e.g., proteins, small interfering RNA) molecules, with presumably minimal side effects.[5] PLGA has a number of advantages over other delivery systems. Phospholipid-based liposomes have stability issues and can be rapidly cleared *in vivo*,[6] and polymersomes are primarily used for short-term release (1 h to a few days).[7–9] PLGA polymer systems can be engineered with a broad range of properties and degradation kinetics that enable sustained release. In addition, porous PLGA foams have been widely investigated as a tissue-engineering scaffold.[10]

Biomaterials can provide a broad range of advantages in medical applications. However, it is important to fully explore potential side effects of implanted materials. PLGA may stimulate inflammation *in vivo* due to the local accumulation of degradation products.[11] However, there has been little investigation of PLGA's effects on cells at the molecular level. Compared to many other macromolecules, DNA is a sensitive molecule and damage may result from exposure to exogenous agents. Prolonged or repeated DNA damage and genomic instability can contribute to multiple diseases including cancer.[12] Therefore, evaluating the impact of PLGA particles on DNA is important. The literature describes several works[13,14] that aimed to determine the toxicity of polymeric nanostructured systems following exposure for 2–48 h. These studies were primarily focused on toxicity related to the size and shape of the materials. In addition,

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TABLE I. Composition of Solutions Used in Generating PLGA Microsphere

	Solvent	Components	Concentration
Inner water phase (w1)	0.2 mL of DI water	BSA	125 mg/mL
		PEG (8,000)	100 mg/mL
		Sucrose	25 mg/mL
Oil phase (o)	1 mL of DCM	PLGA (50:50)	250 mg/mL
		$Mg(OH)_2$	7.5 mg/mL
Outer water phase (w2)	10 mL of DI water	PVA	20 mg/mL

these short-term studies do not address genotoxicity resulting from the degradation of materials such as PLGA, which can occur on the level of weeks.[15] Therefore, we investigated potential genotoxicity over a wider time period.

The aim of this study was to examine the genotoxicity of PLGA microspheres *in vitro* using single cell gel electrophoresis (the comet assay). Two model cell types commonly used in tissue engineering research were examined, human umbilical vein endothelial cells (HUVECS) and human mesenchymal stem cells (MSCs). The comet assay has been shown to be an effective, sensitive, and rapid *in vitro* method for examining DNA damage and issues related to oxidative stress in human cells.[16] The live/dead assay was also performed with HUVECs and MSCs in order to assess time-dependent cytotoxicity and cell viability following exposure to varying concentrations of PLGA microspheres.

MATERIAL AND METHODS

Materials

PLGA 50:50 (Mw = 7,000–17,000, ester terminated), poly(-vinyl alcohol) (PVA, Mw = 13,000–23,000), bovine serum albumin (BSA), sucrose (>99.5%), magnesium hydroxide (95%), PEG (Mn < 3,400), stannous octoate, acryloyl chloride (98%), 3,6-dimethyl-1,4-dioxane-2,5-dione, triethylamine (99.5%), and 2-hydroxy-2-methylpropiophenone (Irgacure 1173) were obtained from Sigma–Aldrich (St. Louis, MO). Dichloromethane (DCM, 99.9%), poly (ethylene glycol) (PEG, Mw = 8,000), sodium chloride (99.5%), magnesium sulfate anhydrous (97%), ethyl ether (anhydrous), and phosphate-buffered saline (PBS) were obtained from Fisher Scientific (Hampton, NH).

Microsphere preparation

A water-in-oil-in-water (w/o/w) double emulsion method[17] was used to generate PLGA microspheres. Two hundred and fifty milligrams of PLGA (50:50) was dissolved in 1 mL of DCM (oil phase, o), with 7.5 mg of $Mg(OH)_2$ added as an anti-acid agent. Twenty five milligrams of BSA, 20 g of PEG (Mw 8,000) and 5 mg of sucrose were dissolved in water to form the water phase (w1), then the oil phase (o) and water phase were mixed by vortex mixing (Fisher Scientific Analog Vortex Mixer; 120 V; speed 10×) for 90 s. The emulsion was then immediately mixed with a 2% PVA solution (w2) to form a water-in-oil-in-water (w1/o/w2) double emulsion and vortexed at 5× speed for 90 s. The double emulsion mixture was then added to 100 mL of deionized (DI) water and stirred overnight to allow organic evaporation and microsphere formation. The microspheres were collected by centrifugation (2,000 × *g*, 10 min), washed twice with DI water, and lyophilized to a dry powder. Components of the PLGA microspheres are shown in Table I.

Polymer synthesis

Synthesis of poly (ethylene glycol)-co-(l-lactic acid) diacrylate (PEG-PLLA-DA) was performed based on the procedures developed by Sawhney et al.[18] All glassware and stir bars were dried in a vacuum oven at 100°C for 24 h prior to use. Ten grams of PEG mixed with 2.12 g of 3,6-dimethyl-1, 4-dioxane-2, 5-dione was lyophilized overnight. The lyophilized PEG, 3,6-dimethyl- 1,4-dioxane-2,5-dione, and 40 μL of stannous octoate were placed in a round-bottomed flask. Vacuum and argon gas cycles were repeated 3× in the reaction vessel to ensure the removal of trace water and oxygen. In order to perform the reaction at a uniform temperature (140°C) the entire flask was submerged in an oil bath and allowed to react for 4 h. The resulting products were dissolved in 40 mL of dichloromethane, filtered with a glass fiber filter (GF/F, Whatman, Maidstone, UK) and precipitated in ice-cold ethyl ether. To acrylate PEG-PLLA, 10 g of PEG-PLLA was lyophilized and placed into a three neck round-bottomed flask with 20 mL of dichloromethane. Two moles of triethylamine per mole of PEG-PLLA was added into the flask and stirred for 5 min under an inert Argon gas. Four moles of acryloyl chloride per mole of PEG-PLLA was added drop wise and reacted overnight in the dark under an inert gas environment. The resulting products were washed with 5 mL of K_2CO_3 (2 M) for inorganic material separation and then precipitated into 1.2 L of ice-cold ethyl ether to remove the residual acryloyl chloride. The products were dissolved in $CDCl_3$ and proton Nuclear Magnetic Resonance ^1H NMR (Advance 300 Hz; Bruker, Billerica, MA) was performed to evaluate structure and purity of the polymer.

Hydrogel preparation

A salt leaching technique was used to prepare porous PEG-PLLA-DA hydrogels.[19,20] Two hundred and fifty milligrams of PEG-PLLA-DA was dissolved in 1 mL of ethanol, with 2-hydroxy-2-methylpropiophenone (0.5% v/v) added as a photoinitiator. Sodium chloride was ground with a pestle and mortar and sieved to select crystals of a defined size range. Four hundred milligrams of salt crystals (300–500 μm) was added per 200 μL of precursor solution polymerized under UV light (k = 365 nm) for 5 min. The hydrogels were then incubated in 50 mL of deionized (DI) water overnight to leach out the salt crystals and ethanol.

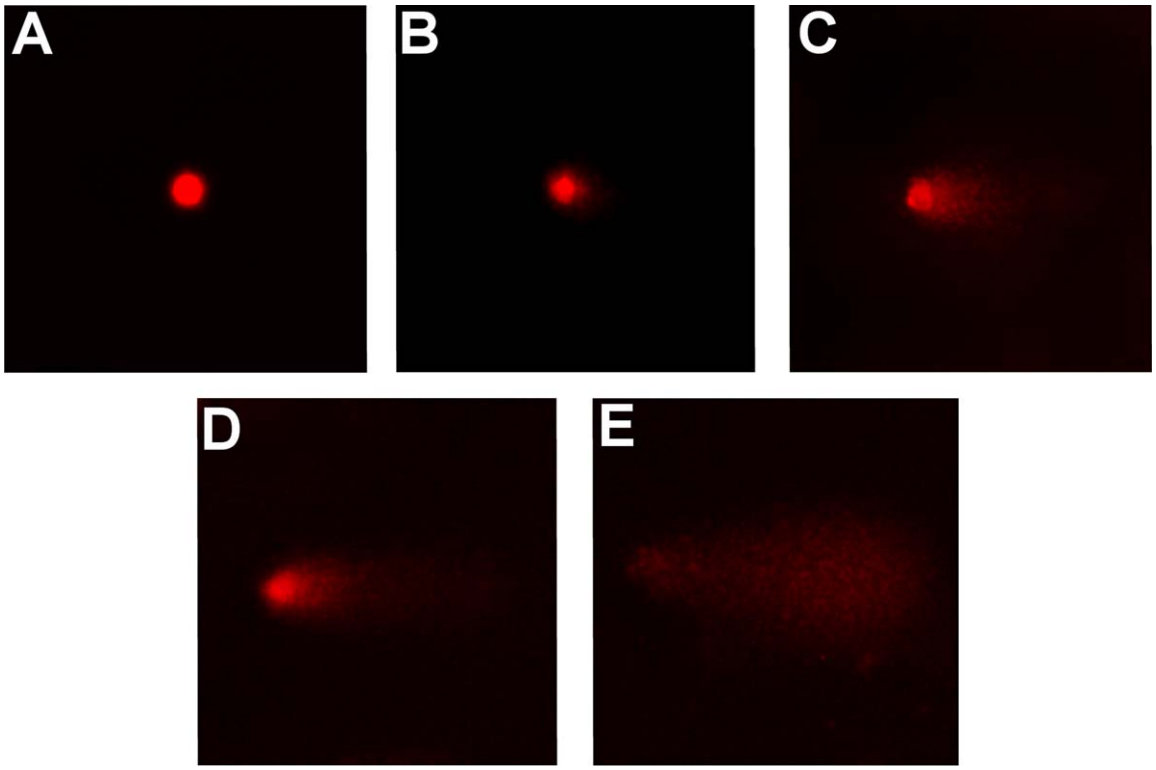

FIGURE 1. Five classes of comets: (A) no damage, <5% (B) low level damage, 5–20% (C) medium level damage, 20–40% (D) high level damage, 40–95% (E) total damage, >95%.

Fifteen milligrams of PLGA was mixed with 50 μL of PEG-PLLA-DA (10%, w/v) and applied on top of the porous hydrogel and polymerized under UV light ($k = 365$ nm) for 5 min.[21,22] Hydrogel with PLGA top layer was placed in the membrane inserts (pore size 0.4 μm) in the 12 transwell plates.

Cell culture

HUVECs (Lonza) were cultured in T-75 flasks with endothelial growth medium (EGM-2, Lonza). The culture medium was changed every 2–3 days. Human mesenchymal stem cells (hMSCs, Lonza) were cultured in T-75 flasks in mesenchymal stem cell growth medium (MSCGM, Lonza) with media refreshed every 3–4 days. All cells were incubated under an atmosphere of 5% CO_2 at 37°C.

When the cells reached approximately 80% confluence, they were dissociated using TrypLE (Gibco) and seeded in 12-well transwell plates at 10,000 cells/well for hMSCs and 5,000 cells/well for HUVECs in 2 mL of complete media. Cells were incubated for 3 h prior to the addition of PLGA microspheres. To test the genotoxicity of PLGA, 1, 5, or 15 mg of PLGA microspheres were suspended in 0.5 mL media and placed within an insert membrane (pore size 0.4 μm; membrane diameter: 12 mm; nominal pore density: 4×10^6 pores per well) that was placed in the well allowing exposure without direct contact. The PLGA doses were selected based on the range of levels previously used for tissue engineering and drug delivery studies.[20,21,23] The final concentrations of PLGA microspheres were 0.4, 2, and 6 mg/mL. Culture medium was changed every 2–3 days within the well only.

HUVECs and hMSCs were exposed to salt leached PEG-PLLA-DA hydrogels with and without a PLGA layer (6 mg/mL) in 12-well transwell plates for 7 days. Culture medium was changed every 2–3 days within the well only. Samples were cultured simultaneously with PLGA groups and a negative control (receiving culture media only).

Cell viability assay

Cell viability was assessed by a live/dead, viability/cytotoxicity kit (Invitrogen). Staining was performed according to the manufacturer's instructions. Cells were incubated for 15 min prior to analysis using confocal microscopy (Carl Zeiss, Oberkochen, Germany). The number of cells with green and red fluorescence was counted in three images per sample (100× magnification, 1.79 μm/pixel). The percentage of cells that exhibited green fluorescence (interpreted as viable cells) was then calculated.

Comet assay

The comet assay was performed as described by Singh et al.[24] The alkaline comet test was used to detect DNA strand breaks and alkali-labile sites. The extent of DNA migration indicates the degree of DNA damage in the cell. After exposure to PLGA, the cells were resuspended and centrifuged. HUVECs were centrifuged 10 min at $200 \times g$ and MSCs were centrifuged for 5 min at $600 \times g$. Approximately 10,000 cells were embedded in 1% low melting-

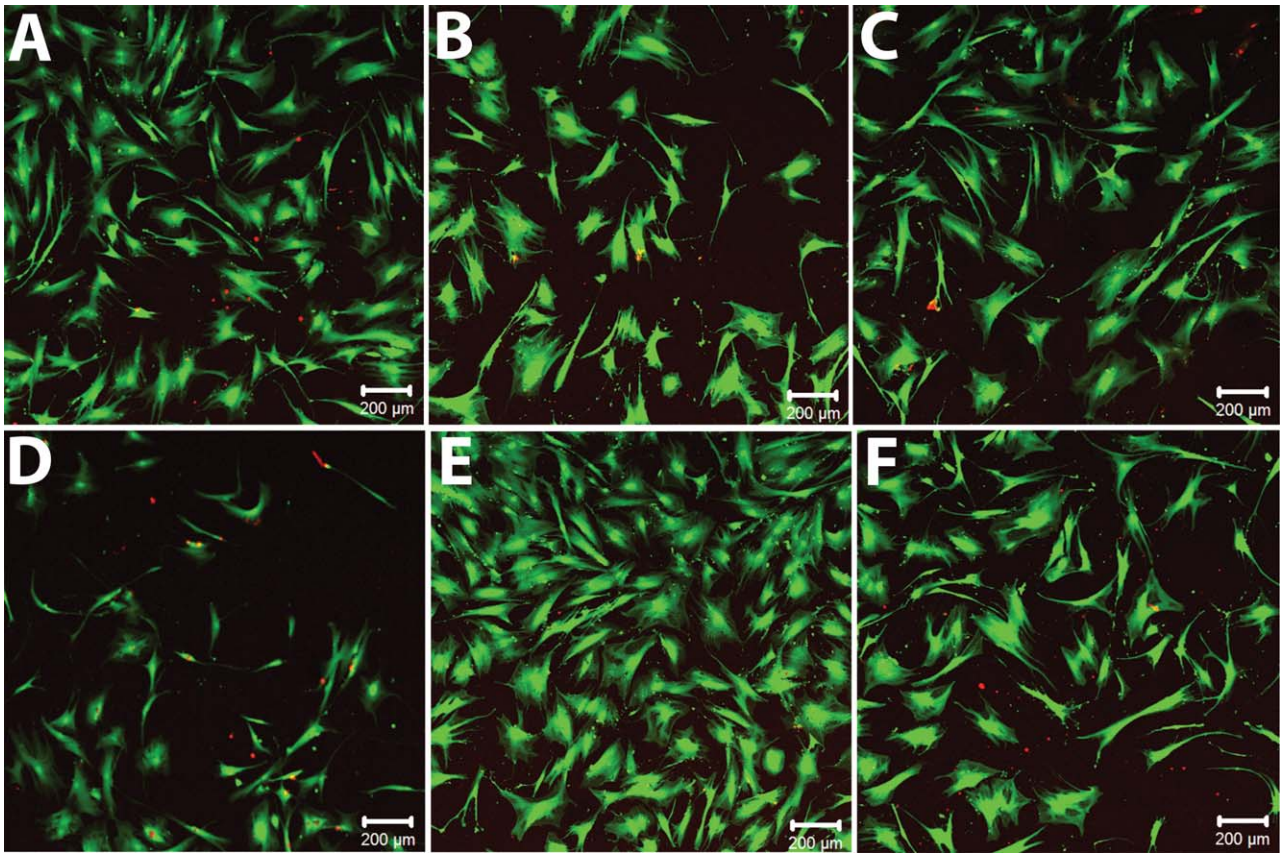

FIGURE 2. Representative images from cytotoxicity test (live dead assay) for MSC after 7 days of exposure to (A) 0.4, (B) 2.0, and (C) 6.0 mg/mL of PLGA microspheres. (D) Control MSCs are shown (blank), in addition to MSCs exposed to (E) hydrogels without PLGA and (F) hydrogels with 6.0 mg/mL PLGA microspheres. *Green indicates live cells and red dead cells.

point (LMP) agarose in PBS, on a glass microscope slide pre-coated with a layer of 1% normal melting-point agarose covered with a glass cover slip and kept for 5 min at 4°C. After gently removing the coverslips, the slides were covered with a third layer of 0.67% LMP agarose and again allowed to solidify for 5 min at 4°C. As a positive control, untreated cells were exposed to 50 μM H_2O_2 for 15 min at 4 °C.

All slides were placed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X100, and 10% dimethylsulfoxide, pH 10 adjusted with NaOH) at 4°C overnight and afterwards subjected to electrophoresis for 20 min at 25 V (300 mA). After neutralization for 15 min in neutralization buffer (0.4 M Tris, pH 7.5), followed by 10 min in water, slides were stained with ethidium bromide (20 lg/L), performed as described by Singh et al.[24] The comets were analyzed at 200× magnification (0.27 μm/pixel) via fluorescence microscopy (Carl Zeiss, Oberkochen, Germany) equipped with a mercury lamp HBO (50 W, 516–560 nm, Zeiss). Evaluation of DNA damage was performed according to Anderson et al.[16] DNA damage in the cells was assessed based on DNA migration from the core of the nucleus and comets were visually scored and classified into five categories corresponding to the extent of DNA migration: (A) no damage, <5%; (B) low level damage, 5–20%; (C) medium level damage, 20–40%; (D) high level damage, 40–95%; (E) total damage, >95% (Fig. 1). Analysis was performed on 100 randomly selected cells per sample and was always carried out by the same experienced person. DNA damage was characterized as any condition where DNA migration over 5% (B + C + D + E comet classes), and the mean value was calculated for three repeated experiments (Fig. 1).

Statistical analysis

Statistical analysis was performed by using Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons posttest for cytotoxicity and genotoxicity evaluation in GraphPad Prism 5.0. Values are expressed as mean ± standard deviation for $n = 3$. A difference at $p < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity

PLGA microspheres were suspended in cell culture medium at a range of concentrations (0.4, 2, and 6 mg/mL) based on levels often used in biomedical applications and placed in a transwell insert (pore size 0.4 μm) enabling exposure to degradation products without direct contact. MSCs and HUVECs were exposed for up to 7 days. When observed

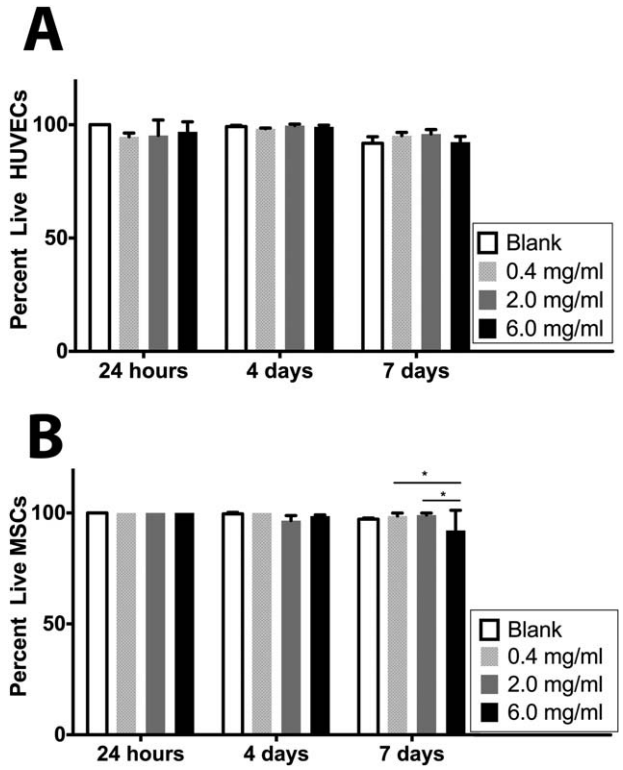

FIGURE 3. The PLGA microspheres do not exhibit significant cytotoxicity for (A) HUVECs or (B) MSCs as determined from analysis of live/dead stains. The percentage of live cells is shown following exposure to PLGA microspheres for 24 h, 4 days, and 7 days. *Denotes statistical significance with $p < 0.05$ by using two-way ANOVA.

under a microscope there was no visual evidence of alterations in cell morphology at any time point or concentration.

Cytotoxicity was examined using a live/dead cell assay. The cells were exposed to both microspheres alone and PEG-PLLA-DA hydrogels (with 6 mg/mL PLGA or without PLGA). The hydrogel delivery strategy represents a model commonly found in tissue engineering where PLGA microspheres are embedded in a biomaterial scaffolds.[20–22] Representative live-dead images for MSCs after 7 days of exposure are shown in Figure 2. In the images, the majority of the cells remain adhered to the surfaces with appropriate morphology. The same was observed with HUVECs exposed to PLGA (data not shown). Image analysis allowed quantification of cell viability under the different conditions. These data support the conclusion that neither PLGA microspheres (Fig. 3) nor hydrogels (Fig. 4) exhibit substantial cytotoxicity for HUVECs or MSCs. There were no differences in cell viability relative to controls at any time point.

Genotoxicity

In the majority of pre-clinical research of biomaterials, the assessment of toxicity is limited to the use of Live/Dead stains and simple image analysis. However, these techniques do not address the more critical concern of genotoxicity. We employed the comet assay to assess DNA damage in MSCs and HUVECs resulting from exposure to PLGA microspheres and PEG-PLLA hydrogels. At a concentration of 0.4 mg/mL the PLGA microspheres did not significantly increase DNA damage (the mean number of cells with DNA migration) in either cell type at any of the times examined (24 h, 4 days, and 7 days) (Fig. 5). While differences were not statistically significant, there was a trend toward an increase in the number of cells with DNA damage cells after exposure to 0.4 mg/mL of PLGA microspheres between 4 and 7 days in HUVECs and between 24 h, 4 and 7 days in MSCs (Fig. 5).

At higher microsphere concentrations (2 and 6 mg/mL) DNA damage was significantly increased in HUVECs and MSCs after 4 and 7 days of exposure. The extent of DNA damage exhibited a dose dependency (Fig. 5). Two mg/mL of PLGA microspheres induced significant DNA damage in HUVECs after 4 (5.33%, $p < 0.01$) and 7 days (7.00%, $p < 0.001$) of exposure compared to controls (1.33), while at 6 mg/mL DNA damage in HUVECs was higher than controls (1.33%) at 4 days (5.66%, $p < 0.01$) and 7 days (8.67%, $p < 0.001$) [Fig. 5(A)].

MSCs exhibited a similar response to the microspheres to what was observed with HUVECs. The levels of DNA damage after 4 days of exposure to 2 (17.60%, $p < 0.01$) and 6 mg/mL (17.60%, $p < 0.01$) of PLGA microspheres was higher than controls (12.30%) [Fig. 5(B)]. The level of DNA damage was higher at 7 days, with the percentage of MSCs exhibiting DNA damage as 19.60% (2 mg/mL, $p < 0.001$) and 24.60% (6 mg/mL, $p < 0.001$) with controls only at 12.00%. At 6 mg/mL PLGA the DNA damage in MSCs was higher than 2 mg/mL after 7 days ($p < 0.05$).

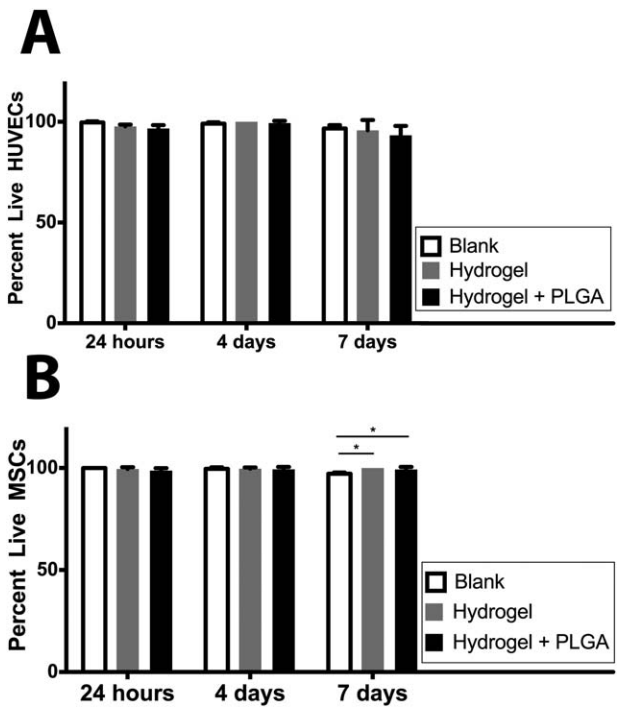

FIGURE 4. The PEG-PLLA hydrogels did not exhibit significant cytotoxicity with or without PLGA microspheres. Percent viability for (A) HUVECs and (B) MSCs determined from analysis of live/dead stains. The percentage of live cells is shown following exposure for 24 h, 4 days, and 7 days. *Denotes statistical significance with $p < 0.05$ by using two-way ANOVA.

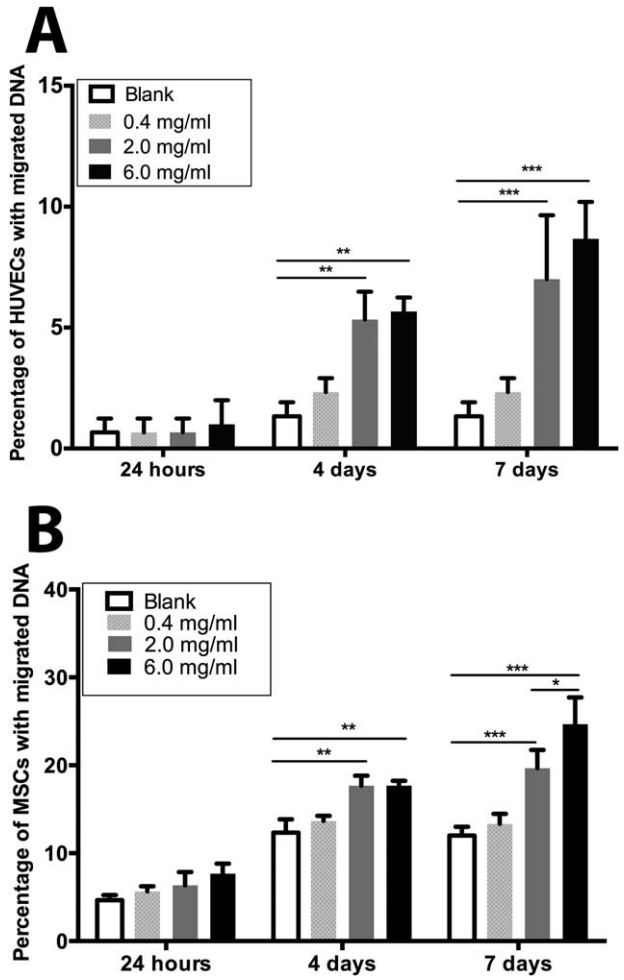

FIGURE 5. PLGA microspheres induces dose and time dependence increases in DNA damage. The percentage of (A) HUVECs and (B) MSCs exhibiting DNA damage after exposure to PLGA microspheres of 0.4, 2 and 6 mg/mL during 24 hours, 4 days and 7 days are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by using *two-way ANOVA* test.

We next examined whether exposure of HUVECs and MSCs to PEG-PLLA-DA hydrogels with or without PLGA microspheres induced DNA damage. This approach is a model of common strategies employed in tissue engineering and has been used in our laboratory to influence vascularized tissue formation.[20,21] The PEG-PLLA-DA copolymer hydrogel did not induce DNA damage in the absence of PLGA microspheres with either HUVECs or MSCs at 24 h, 4 days or 7 days (Fig. 6). No significant DNA damage was observed during 24 h of exposure of either HUVECs or MSCs to hydrogels containing 6 mg/mL of PLGA microspheres. However, following longer exposures both cell types showed significantly higher levels of DNA damage. The levels of DNA damage in HUVECs were higher at 4 (6.33%) and 7 days (6.67%) relative to controls (1.33% and 1.67%, $p < 0.001$) [Fig. 6(A)]. Higher DNA damage was also seen in after 4 (24.00%) and 7 days (29.30%) of exposure to the hydrogels containing microspheres. These values were significantly higher than controls (11.33% and 12.67%, $p < 0.001$) [Fig. 6(B)].

DISCUSSION

PLGA microspheres have been studied extensively as drug delivery systems and as scaffolds for tissue engineering. In order to fully understand risks associated with their clinical application, potential side effects should be evaluated. DNA damage caused by both exogenous and endogenous agents can lead to degenerative changes and result in cell transformation that may progress to diseases such as cancer.[25] In this study, DNA strand breaks and levels of unrepaired cellular DNA damage were measured using a simple and reproducible technique, the comet assay.

The two cell types studied here, HUVECs and MSCs, are widely used as model cells in the evaluation of biomaterials and tissue engineering products.[26,27] Results of the DNA comet assays indicate that PLGA concentrations of 2 and 6 mg/mL induce DNA damage in both types of cells when exposed for 4 days or more. PLGA degradation requires a time period of 1–5 weeks;[15] it appears that after 4 days the levels of degradation products are high enough to induce

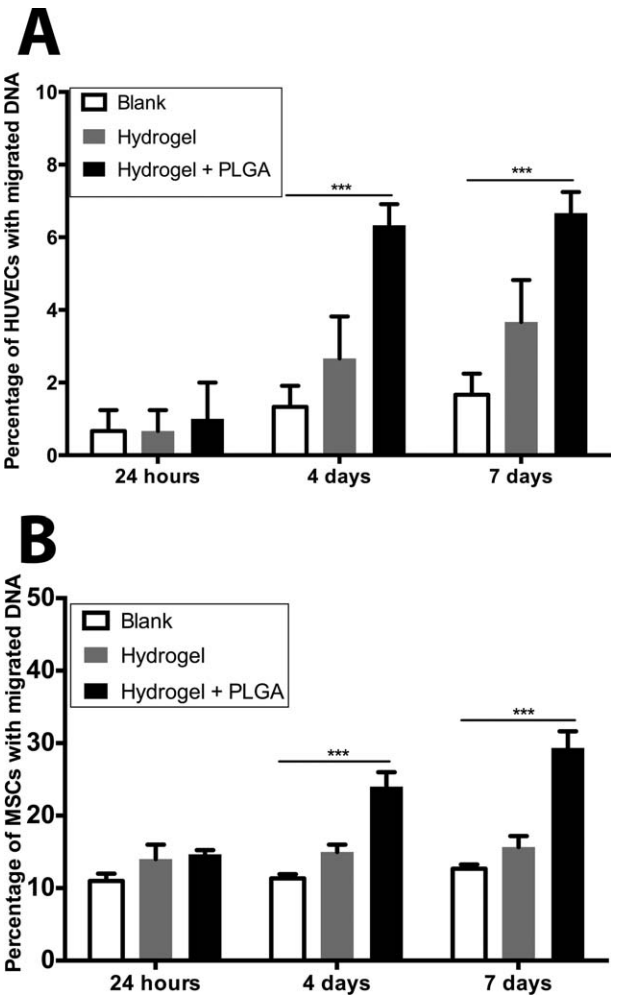

FIGURE 6. PEG-PLLA-DA hydrogels do not induce DNA damage but combination of hydrogels with PLGA microspheres elevates the level of DNA damage. The percentage of (A) HUVECs and (B) MSCs exhibiting DNA damage following exposure to PEG-PLLA-DA hydrogels and hydrogels with PLGA (6 mg/mL) at 24 h, 4 days, and 7 days. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by using *two-way ANOVA* test.

damage. The degradation products at higher concentrations may interact with nuclear DNA (directly or indirectly), producing significant DNA damage. In the view of substantially, more DNA damage cells at 6 mg/mL relative to 2 mg/mL during 7 days of exposure on MSCs, we hypothesized that damaged cells did not have enough capacity to repair DNA damage in cells induced by 6 mg/mL PLGA concentration compared to 2 mg/mL.

PLGA is a biodegradable polymer, which undergoes hydrolysis, producing lactic acid and glycolic acid that are readily metabolized in organisms. The human body excretes them by a glycolytic route in the form of carbon dioxide and water. It is generally believed that degradation of PLGA is a slow process that does not interfere with cellular metabolism.[25,28,29] However, our data suggested that PLGA degradation products can lead to structural changes of DNA molecules. Kornhauser et al.[30] showed that human skin cells treated with glycolic acid (10%) had increased UV-sensitivity and induces DNA damage. It has also been shown that PLGA-PEO nanoparticles significantly increased the level of micronuclei (MN) in mononucleated cells suggesting early aneugenic effects of weak mutagens while 0.12–75 μg/cm^2 of PLGA-PEO nanoparticles during 2 and 24 h exposure did not induced DNA damage.[13] This significant olychromatic erythrocytes with micronuclei were not identified in an *in vivo* study by Göelzer et al.[31] At a low concentration (0.4 mg/mL) exposure to PLGA microspheres did not exert any significant DNA damaging effects over the tested time frames (24 h–7 days). This is in agreement with the reports by De Lima et al.[28] and Setyawati et al.[14] The comet assay results show that hydrogels under the conditions of this study did not induce DNA migrations in both types of cells, suggesting that hydrogel is not potentially genotoxic. On the other hand, PEG-PLLA-DA hydrogel with PLGA significantly induced a damaging effect on DNA after 4 and 7 days of exposure.

Our results should contribute to the enlightening of PLGA degradation as a cell-biodegradable material and its impact on cellular biomolecules, particularly genomic material. DNA damage increases with degradation time, and degradation of PLGA is faster *in vivo* compared to that *in vitro*.[32,33] According to these findings, it is necessary to investigate the effect of PLGA microsphere degradation in *in vivo* systems. Regardless, the comet assay only indicates DNA damage and possible mutagenesis. Additional studies are needed in order to further examine any potential mutagenic effects.

CONCLUSION

PLGA-based materials are under investigation for a broad range of applications. These studies show that exposure of cells to PLGA microspheres can result in DNA damage that varies with concentration and duration of exposure. While DNA damage does not necessarily mean mutagenesis, the mechanism underlying this damage needs to be further examined to fully understand the phenomenon and associated risks.

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