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Alireza Rahimnejad Yazdi

Lawrence Torkan

Stephen D. Waldman

Mark R. Towler
Missouri University of Science and Technology, mtowler@mst.edu

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Development of a novel bioactive glass suitable for osteosarcoma-related bone grafts

Alireza Rahimnejad Yazdi,[1,2] Lawrence Torkan,[1,3] Stephen D. Waldman,[2,4] Mark R. Towler[1,2]

[1]Department of Mechanical and Industrial Engineering, Ryerson University, Toronto, Ontario M5B 2K3, Canada
[2]Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, Ontario M5B 1W8, Canada
[3]Faculty of Science, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada
[4]Department of Chemical Engineering, Ryerson University, Toronto, Ontario M5B 2K3, Canada

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Abstract: In this study, zinc borate-based glasses with increasing gallium content (0, 2.5, 5, 10, and 15 wt % Ga) were synthesized and their effect on the viability and proliferation of preosteoblasts and osteosarcoma cancer cells were investigated. Methyl thiazolyl tetrazolium (MTT) cell viability assays using glass degradation extracts revealed that the extracts from glasses with lower Ga contents could enhance the viability of preosteoblasts, while extracts from the glass composition with 15 wt % Ga caused statistically significant reduction of their viability. MTT cell viability assays using the extracts and osteosarcoma cells showed that only extracts from the glass composition with 5 wt % Ga (G3) did not cause a statistically significant increase in the viability of cancer cells for all degradation periods (1 day, 7 days, and 28 days). G3 was selected as the most suitable composition for the osteosarcoma-related graft operations as it could improve the viability of preosteoblasts without increasing the viability of cancer cells. The viability of preosteoblasts and osteosarcoma cells in contact with the glass powders were also investigated using MTT assays. The results showed that the G3 powders could enhance the viability of preosteoblasts while decreasing the viability of osteosarcoma cells. Finally, live/dead assays revealed that suppression of proliferation appeared to be the mechanism causing the observed reductions in the viability of osteosarcoma cells exposed to G3 powders. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 106B: 1186–1193, 2018.

Key Words: gallium, zinc borate glass, preosteoblast, osteocarcoma, cell viability

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INTRODUCTION

Bioactive glasses have potential for orthopedic applications. They can be formulated to accelerate the regeneration of bone tissue that has undergone trauma or disease. This presents a favorable alternative to live tissue donation, which is in limited supply.[1–3] Moreover, bioactive glasses' formulation can be altered such that they deliver therapeutic ions to the body as they degrade. Among the possible elements that can be added to the bioactive glasses' formulation, gallium has shown to be a very promising candidate due to its antibacterial properties, ability to suppress bone resorption, and its antineoplastic properties in ionic form.[4–6]

The gallium ion works to combat cancer cells through a variety of mechanisms of physiological interference, mostly involving DNA replication and disruption of its helical structure. It is considered a non-functional mimetic of iron (Fe) and can replace iron in transferrin to inhibit ribonucleotide reductase, a key enzyme involved in the replication of DNA.[7] Its effectiveness stems from direct inhibition of the enzyme and blocking the supply of iron available to bind to the enzyme for proper functioning.[8] Gallium also competes with magnesium ions, which stabilize the DNA structure and aid in the binding of specific nucleic acid-associated proteins. It has been shown that the affinity of gallium for DNA is 100 times higher than that of magnesium.[9] When higher concentrations of gallium ion are present *in vitro* with a high Ga: DNA ratio ($\geq$1/40), it has been reported that destabilizing reactions occur by means of gallium bonding to the nucleic bases.[10] In addition to vying with various ions, gallium has the ability to induce chromatin condensation *in vitro*. This is considered an early step in the process of apoptosis and results in cell death through a mechanism of Fe deprivation.[11]

With respect to bone metabolism, the gallium ion has been extensively studied due to its marked ability to decrease hypercalcemia associated with cancer.[12–15] Its physiological effects consist of inhibition of osteoclastic activity, an increase in collagen synthesis correlating to intraosseous concentrations of Ga, as well as an increase of bone tissue formation *in vitro*.[16–21] Osteoclast resorption is inhibited through alteration of gene expression of a bone matrix signaling protein.[22] Exposure of mesenchymal or mesenchymally derived cells to Ga also causes a change in matrix protein synthesis to favor

Correspondence to: A. Rahimnejad Yazdi; e-mail: alireza.rahimnejadya@ryerson.ca
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TABLE I. The Nominal Compositions (wt %) of the Glasses

	Glass1 (G1)	Glass2 (G2)	Glass3 (G3)	Glass4 (G4)	Glass5 (G5)
B_2O_3	52	49.5	47	42	37
ZnO	16	16	16	16	16
Na_2O	14	14	14	14	14
CaO	12	12	12	12	12
P_2O_5	6	6	6	6	6
Ga_2O_3	0	2.5	5	10	15

bone formation through a similar mechanism at the level of gene expression.[23] An additional benefit of inducing tissue fibrosis in the tumor[24,25] and evaluation of vascular permeability have also been observed.[26] Radioactive isotopic compounds have shown promise in their absorption by cancerous deposits in bone.[27] Ga-67 scans can also be used to predict the effectiveness of this treatment by scanning and observing the degree of visibility of tumors following absorption of the isotope.[28]

The abovementioned therapeutic properties of gallium explain the motives behind recent studies around its incorporation into bioactive glasses.[29–31] These glasses appear particularly beneficial for the treatment of patients with osteoporosis, bone cancer, and malfunctioning immune systems. It has been hypothesized that glass grafts that release gallium ions can reduce or eliminate the removal of healthy bone tissue that is currently extracted as part of the tumor removal surgery for bone cancer.[30,31] However, this hypothesis has not yet been investigated experimentally. Previously, a series of novel gallium containing zinc borate glasses were developed and their chemical structure, degradation behavior in both water and SBF, and antibacterial properties were evaluated.[32,33] In this study, for the first time, the effect of gallium-releasing bioactive glasses on bone cancer cells has been investigated and the results were compared their effect on healthy bone cells.

MATERIALS AND METHODS

Glass synthesis and preparation

Five compositions of borate glasses with increasing gallium content (0, 2.5, 5, 10, and 15 wt % Ga), added at the expense of boron, were formulated (Table I). Appropriate amounts of analytical grade reagents (boron oxide, sodium carbonate, calcium carbonate, ammonium dihydrogen phosphate, zinc oxide, gallium oxide; Fisher Scientific, Ottawa, ON, Canada; Sigma-Aldrich, Oakville, ON, Canada) were weighed out in a plastic tub and mixed into a container for 15 min to obtain a homogenous mix of all reagents. The mixes were dried in the oven at 100°C for 1 h, and then transferred to silica crucibles for firing (1200°C, 1 h). The

TABLE II. D10, D50, and D90 Values in Micrometers for Glass Powders Used in Ion Release Experiments

	G1			G2			G3			G4			G5		
	D10	D50	D90	D10	D50	D90	D10	D50	D90	D10	D50	D90	D10	D50	D90
	95	141	271	101	152	277	97	143	282	96	147	279	98	149	276
STD	4.58	7.54	12.76	1.73	4.35	8.54	3.6	8.18	8.71	4.35	11.13	9.64	3.46	7.93	13.22

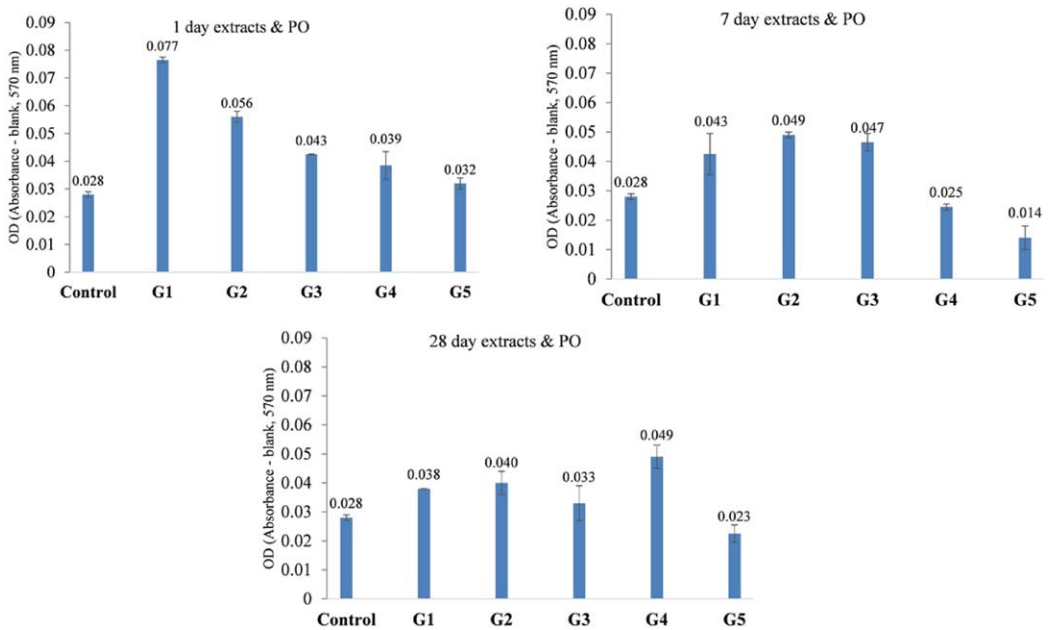

FIGURE 1. The viability of the preosteoblast cells in the presence of 1 day, 7 day, and 28 day glass extracts.

TABLE III. The *p* Values for the PO Cell Viability Measurements of the Glass Extracts According to Mann–Whitney *U* Test

	C, G1	C, G2	C, G3	C, G4	C, G5	G1, G2	G1, G3	G1, G4	G1, G5	G2, G3	G2, G4	G2, G5	G3, G4	G3, G5	G4, G5
P_{1day}	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.376	0.05	0.127
P_{7days}	0.05	0.05	0.05	0.05	0.05	0.275	0.376	0.05	0.05	0.275	0.05	0.05	0.05	0.05	0.05
P_{28days}	0.037	0.05	0.376	0.05	0.05	0.487	0.487	0.037	0.037	0.127	0.05	0.05	0.05	0.05	0.05

TABLE IV. The Concentration (ppm) of the Released Ions From Glass Powder After Soaking in DI Water for 1, 7, and 28 days at 37°C

	1 day			7 days			28 days		
	Ga	Zn	Ca	Ga	Zn	Ca	Ga	Zn	Ca
G1	0	0.04	62	0	0.18	162	0	0.46	195
G2	33	0.04	68	49	0.16	145	61	0.40	146
G3	75	0.02	57	101	0.15	91	123	0.33	141
G4	107	0.02	38	179	0.14	119	196	0.29	152
G5	181	0.01	46	276	0.08	128	285	0.24	131

glass melts were shock quenched into water and the resulting frits were dried, ball milled, and sieved to retrieve glass powders with particle sizes of 90–710 μm.

Glass degradation extracts

Glass powders were soaked in distilled water (with the ratio of 0.50 g powders to 50.0 mL solution[34]) for 1, 7, and 28 days at 37°C. Glass particles within the size range of 90–710 μm and D10, D50, and D90 values shown in Table II were used as samples. The samples were filtered after 1, 7, and 28 days of soaking in deionized water.

The filtered solutions were tested by inductively coupled plasma atomic emission spectroscopy (ICP-AES) to measure the concentration of released ions. Five calibration standards were prepared for each element and deionized water was used as control. Measurements were repeated three times for each sample.

MTT cell viability tests

Cytotoxicity of the glasses' degradation extracts. Cell viability assays using the methyl thiazolyl tetrazolium (MTT) kits were conducted to determine *in vitro* cytotoxicity of the glasses. Preosteoblastic MC3T3-E1 (ATCC CRL-2593) and SaOS2 Osteosarcoma (ATCC® HTB-85™) cells were used for this study. Cells were seeded into 24 well plates at a density of 10,000 cells per well in minimum essential medium alpha (MEMα) supplemented with 10% fetal bovine serum and 1% (2 mM) L-glutamine (Cambrex, MD, USA) and maintained in a cell culture incubator at 37°C/5% CO_2. Twenty-four hours after seeding, 100 μL of liquid extract (from the degradation samples at 1, 7, and 30 days for all glasses) was added into the wells containing the cells in culture medium (1 mL) and further cultured for 24 h. The MTT was added in an amount equal to 10% of the culture medium volume/well. The cultures were then reincubated for a further 2 hours (37°C/5% CO_2) after which the cultures were removed from the incubator and the resultant formazan crystals were dissolved by adding an amount of MTT solubilization solution (10% Triton X-100 in acidic isopropanol (0.1 N HCl)) equal to the original culture medium volume. Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm. Control media and cells cultured in absence of liquid extracts were used as a reference. This cytotoxicity experiment was repeated in triplicate for degradation extracts of each glass composition and degradation period.

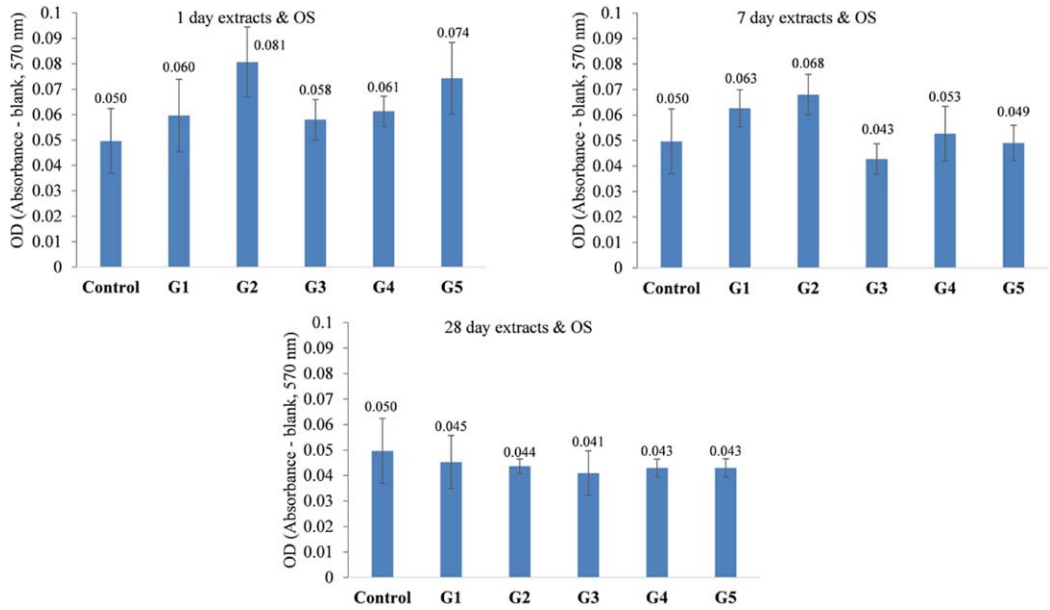

FIGURE 2. The viability of the osteosarcoma cells in the presence of 1 day, 7 day, and 28 day glass extracts.

Cytotoxicity of the glass powders. After conducting cytotoxicity tests with the glasses' extracts, G3 glass formulation was selected for further analysis. 1, 2, and 5 mg of G3 glass powders were weighed (METTLER TOLEDO XP26, Max 22 g, $d = 0.001$ mg) and sprinkled on the bottom of the wells. Then, preosteoblastic MC3T3-E1 (ATCC CRL-2593) and $SaOS_2$ Osteosarcoma (ATCC® HTB-85™) cells were seeded into the well plates at a density of 10,000 cells per well in minimum essential medium alpha (MEMα) supplemented with 10% fetal bovine serum and 1% (2 mM) L-glutamine (Cambrex, MD, USA) and cultured for 48 h. The MTT was added in an amount equal to 10% of the culture medium volume/well. The cultures were then reincubated for a further 2 h (37°C/5% CO_2) after which the cultures were removed from the incubator and the resultant formazan crystals were dissolved by adding an amount of MTT solubilization solution (10% Triton X-100 in acidic isopropanol (0.1 N HCl)) equal to the original culture medium volume. Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm. Control media and healthy growing cell population were used as a reference. This cytotoxicity experiment was repeated in triplicate for G3 glass powders.

Live/dead cell viability

Fluorescence-based live–dead assay (Molecular Probes Inc. OR, USA) with calcein AM (for labeling live cells) and EthD-1 (for labeling dead cells) were used to evaluate the viability of the preosteoblastic MC3T3-E1 (ATCC CRL-2593) and $SaOS_2$ Osteosarcoma (ATCC® HTB-85™) cells after exposure to G3 glass powders. First, adherent cells were cultured with 1, 2, and 5 mg of glass powders in 24-well plates as confluent monolayers. Cells were then washed with PBS to remove serum esterase activity in growth media. Next, 100 μL of the combined live/dead assay reagents with standard concentrations (2 μM calcein AM and 4 μM EthD-1) were added directly to the wells to cover all cells. Then the cells were incubated for 45 min at room temperature. After incubation, wells were rinsed with PBS and labeled cells were viewed under microscope (Zeiss Axio Observer A1).

Statistical analysis

The nonparametric Kruskal–Wallis H test and Mann–Whitney U test were used to analyze the data, compare the relative means, and report any statistically significant differences when $p \leq 0.05$. Statistical analysis was performed on all groups where $3 \leq n \leq 9$. Statistical analysis was performed using SPSS software (IBM SPSS statistics 21, IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Preosteoblast (PO) cells were initially tested with degradation extracts obtained by immersion of the glass particles containing varying gallium concentrations (G1–G5) in deionized water for assorted lengths of time. The results from the cell viability test, determined by MTT assays, are presented in Figure 1. The goal of this test was to determine the glass compositions with biologically feasible gallium concentrations to allow for viability of preosteoblasts. As seen in Figure 1, the average number of viable cells generally declined as gallium content increased. For 1 day extracts, all glass compositions did allow for better cell viability compared to the control; for 7 day extracts, G1–G3 compositions allowed for better cell viability compared to the control; and for 28 day extracts, G1–G4 were found to increase cell viability with respect to the control. It is possible that the G5 extracts (7 days and 28 days) contained levels of gallium that surpassed the toxic level in preosteoblasts. This would explain the reduced cell viability in G5 extract relative to

TABLE V. The *p* Values for the OS Cell Viability Measurements of the Glass Extracts According to Mann–Whitney *U* Test

	C, G1	C, G2	C, G3	C, G4	C, G5	G1, G2	G1, G3	G1, G4	G1, G5	G2, G3	G2, G4	G2, G5	G3, G4	G3, G5	G4, G5
P_{1day}	0.268	0.046	0.121	0.046	0.046	0.127	0.827	0.513	0.513	0.127	0.127	0.127	0.275	0.275	0.376
P_{7days}	0.046	0.046	0.825	0.268	0.5	0.275	0.05	0.275	0.046	0.05	0.184	0.046	0.275	0.268	0.825
P_{28days}	0.825	0.5	0.825	0.5	0.825	0.817	0.827	0.825	0.827	1	0.361	0.825	1	0.827	0.825

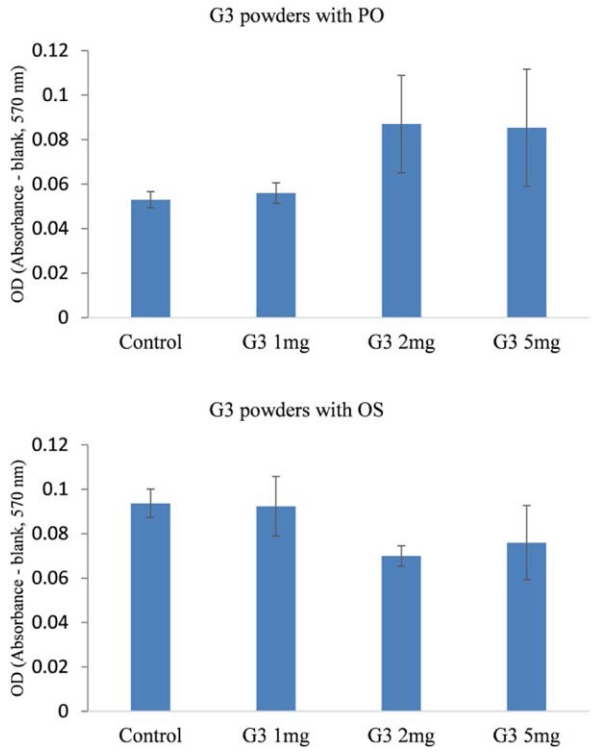

FIGURE 3. Cell viability of PO and OS cells in the presence of 1, 2, and 5 mg of G3 glass powders.

the control media. According to Kruskal–Wallis H test, the measurements for preosteoblast cell viability in different extracts for each incubation time were significantly different ($p \leq 0.05$): $p_{1day} = 0.007$, $p_{7days} = 0.009$, and $p_{28days} = 0.012$. The statistical significance between the means for each incubation time was compared using Mann–Whitney *U* test (Table III). The Mann–Whitney *U* test results confirm that the observed reductions in the preosteoblast cell viability for all G5 extracts (1 day, 7 days, and 28 days) with respect to control samples were statistically significant.

At nontoxic levels, gallium-containing substances have been studied and proved to have a number of benefits with respect to bone metabolism. Gallium nitrate has been used in both *in vivo* and *in vitro* models for the purpose of inhibiting bone resorption without cytotoxic effects on bone cells.[17,19–21,35] Studies conducted by means of gallium-treated rat calvaria suggest that gallium ions may act directly on osteoblasts at the level of gene expression. It was found that treatment with gallium nitrate can suppress stimulation of osteocalcin (OC), an abundant protein synthesized by osteoblasts; reduction of synthesis of OC *in vivo* is associated with enhanced mineralization.[23,36] Osteocalcin has been hypothesized to serve as a signal molecule synthesized by osteoblasts in order for resorption to commence.[22,37–39] Suppression of resorption would indicate to the cell that collagen and bone formation is favored, thus promoting preosteoblast proliferation. As it has been shown that gallium affects protein synthesis related to the fully differentiated osteoblast phenotype, it is possible that optimal levels of gallium could contribute to the enhancement of preosteoblast proliferation and differentiation.

TABLE VI. The *p* Values for the PO and OS Cell Viability Measurements of the Glass Powders (Mann–Whitney *U* Test)

	C, 1 mg	C, 2 mg	C, 5 mg	1 mg, 2 mg	1 mg, 3 mg	2 mg, 3 mg
$P_{G3,\ PO}$	0.275	0.05	0.05	0.05	0.05	0.827
$P_{G3,\ OS}$	0.827	0.05	0.184	0.05	0.275	0.513

Moreover, the viability of the preosteoblast cells could be affected by the other ions released from the glasses. A key ion in the extracts that is involved in bone metabolism is zinc. It plays an active role in stimulating bone formation and has been shown to be concentrated in osteoid prior to its calcification to bone.[40–43] Studies conducted on *in vivo* models confirm that dietary zinc enhances osteoblast differentiation while inhibiting osteoclastic differentiation and subsequent resorption.[44–47] Zinc also enhances ATPase activity, and regulates transcription of genes, such as osteocalcin, osteopontin, ALP, and collagen I, that are responsible for differentiation of osteoblastic cells.[48–50] Calcium ions can also affect osteoblastic cell viability. Calcium ions are reported to intensify osteoblast proliferation; they can activate Ca-sensing receptors in osteoblasts and enhance the expression of growth factors such as IGF-I and IGF-II.[50–53] The ICP measurements of the abovementioned ions in the degradation extracts are shown in Table IV.

The viability of osteosarcoma (OS) cells in the presence of the degradation extracts of the glasses was also investigated using MTT assays. As seen in Figure 2, all 1 day degradation extracts allowed for enhanced viability of the osteosarcoma cells compared to the control media. For 7 day extracts, only those from G3 glass could suppress the proliferation of the osteosarcoma cells. For 28 day extracts, G2–G5 could suppress the proliferation of the osteosarcoma cells. According to Kruskal–Wallis *H* test, the measurements for osteosarcoma cell viability in different extracts for each incubation time were not significantly different ($p \geq 0.05$): $p_{1\text{day}} = 0.087$, $p_{7\text{days}} = 0.051$, and $p_{28\text{days}} = 0.997$. The statistical significance between the means for each incubation time was compared using Mann–Whitney *U* test (Table V). The Mann–Whitney *U* test results show that the observed increase in the osteosarcoma cell viability (compared to the control media) for 1 day G2, G4, and G5 extracts, and 7 day extracts of G1 and G2 were statistically significant.

Considering the results of the conducted MTT assay with glass extracts on both preosteoblast and osteosarcoma cells, the G3 extract was the only one that did not produce a statistically significant rise in the viability of osteosarcoma cells. At the same time, 1 day and 7 day extracts of G3 composition could significantly enhance the viability of preosteoblast cells, while its 28 day extract did not suppress the

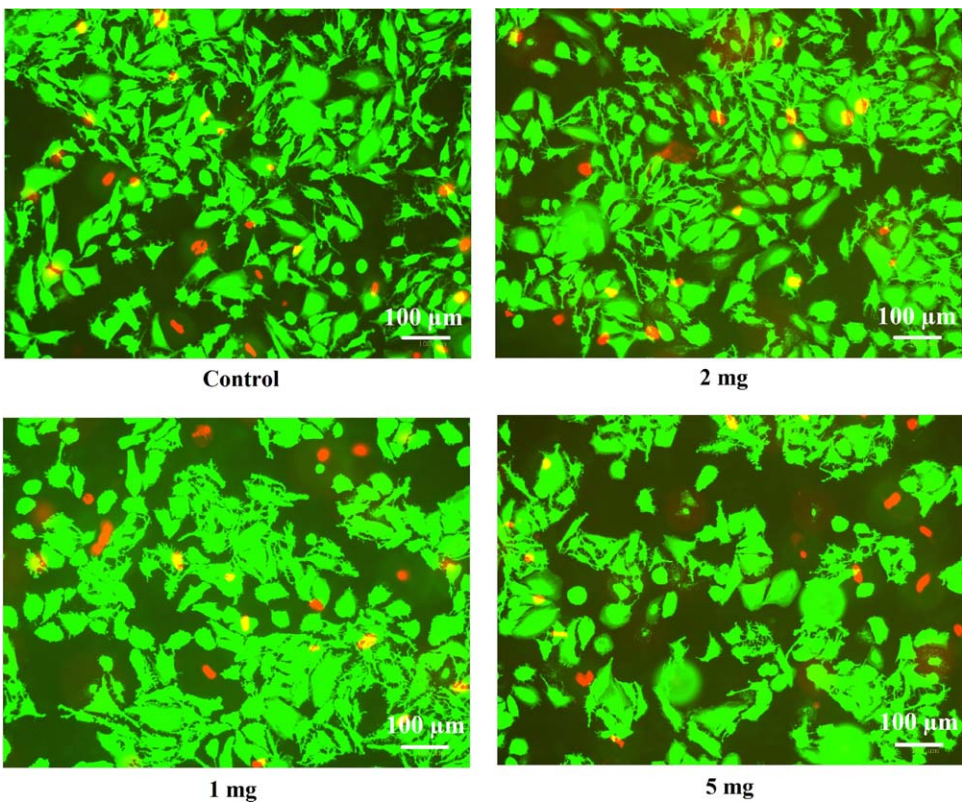

FIGURE 4. Labeled live (green) and dead (red) OS cells after exposure to 1, 2, and 5 mg G3 glass powders.

proliferation of preosteoblasts. Therefore, G3 was chosen for further studies using the glass powders.

MTT assays were used to study the viability of preosteoblast and osteosarcoma cells in the presence of G3 glass powders. Samples at masses of 1, 2, and 5 mg of each glass composition were assayed with both preosteoblast and osteosarcoma cells (Figure 3). It was found that the most effective quantity of the G3 sample, with the criteria of promoting the viability of osteoblasts and suppressing that of osteosarcoma cells, was between 2 and 5 mg. According to Kruskal–Wallis *H* test, the measurements for the viability of preosteoblast cells exposed to different glass powder weights for G3 composition were significantly different ($p \leq 0.05$): $p_{G3,\ PO} = 0.034$. However, the measurements for the cell viability of osteosarcoma cells for various glass powder weights were not statistically significant ($p \geq 0.05$): $p_{G3,\ OS} = 0.114$.

The statistical significance between the means for various powder masses were compared using Mann–Whitney *U* test (Table VI). The comparison of the means showed that increasing the powder mass in contact with the osteosarcoma cells could lead to statistically significant reduction in their viability.

To gain a better understanding of the mechanism causing the observed reduction in the viability of osteosarcoma cells by G3 powders (suppression of proliferation versus induction of apoptosis), fluorescence-based live–dead assay, with calcein AM for labeling live cells and EthD-1 for labeling dead cells, were conducted. As shown in Figure 4, the number of dead osteosarcoma cells stays at the same level for samples exposed to various amounts of G3 glass powders and control media. However, the number of live osteosarcoma cells drops by increasing the amount of G3 glass powders, suggesting that suppression of cell proliferation appears to be the mechanism behind the observed reduction in the cell viability values of osteosarcoma cells exposed to G3 glass powders.

CONCLUSION

The suitability of a novel series of gallium-releasing zinc borate bioactive glasses for osteosarcoma-related bone graft operations were investigated using MTT and live/dead assays. First, the MTT assays were conducted with preosteoblasts and osteosarcoma cells in the presence of glass degradation extracts. G3 extract was the only one that did not cause a statistically significant increase in the viability of osteosarcoma cells. At the same time, 1 day and 7 day extracts of G3 significantly improved the viability of preosteoblast cells, while its 28 day extract did not suppress the viability of preosteoblast cells. Therefore, the composition of G3 was selected for further analyses. Next, MTT assays were conducted to study the effect of the G3 glass powders on the viability of both preosteoblast and osteosarcoma cells. The results indicated that G3 powders could also enhance the viability of preosteoblasts while reducing that of osteosarcoma cells. Finally, the performed fluorescence-based live–dead assay on osteosarcoma cells that were exposed to G3 powders indicated that suppression of proliferation, not induction of apoptosis, was responsible for the observed reduction in the viability of osteosarcoma cells.

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