Glycyrrhizin mitigates inflammatory bone loss and promotes expression of senescence-protective sirtuins in an aging mouse model of periprosthetic osteolysis

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Abstract

Although periprosthetic osteolysis induced by wear debris particles is significantly elevated in senior (65+ years old) patients, most of the published pre-clinical studies were performed using young (less than three-month old) mice indicating the critical need to employ experimental models of particle-induced osteolysis involving mice with advanced age. Emerging evidence indicates that currently available antiresorptive bone therapies have serious age-dependent side effects. However, a resurgence of healthcare interest has occurred in glycyrrhizin (GLY), a natural extract from the licorice roots, as alternative sources of drugs for treating inflammatory bone lytic diseases and prevention of cellular senescence. This study investigated the effects of GLY on inflammatory bone loss as well as expression patterns of senescence-associated secretory phenotype and senescence-protective markers using an experimental calvarium osteolytic model induced in aged (twenty-four-month-old) mice by polymethylmethacrylate (PMMA) particles. Our results indicate that local treatment with GLY significantly diminished the size of inflammatory osteolytic lesions in aged mice via the number of CXCR4+OCPs and Tartrate-resistant acid phosphatase positive (TRAP+) osteoclasts. Furthermore, GLY dramatically decreased the amounts of senescence-associated secretory phenotype markers, including pro-inflammatory macrophage migration inhibitory factor (MIF) chemokine, and cathepsins B and K in the bone lesions of aged mice. By contrast, GLY significantly elevated expression patterns of senescence-protective markers, including homoeostatic stromal derived factor-1 (SDF-1) chemokine, and sirtuin-1, and sirtuin-6, in the PMMA particle-induced calvarial lesions of aged mice. Collectively, these data suggest that GLY can be used for the development of novel therapies to control bone loss and tissue aging in senior patients with periprosthetic osteolysis.

Keywords
Aging; Glycyrrhizin; Particle-induced osteolysis; MIF; Cathepsins; Sirtuins

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Conflict of interest
The authors declare that there are no conflicts of interest.
1. Introduction

Total joint arthroplasty (TJA) is one of the success stories of modern medicine because it is an effective and common procedure to treat the last stages of osteoarthritis in senior (>65 years old) patients [1–3]. However, approximately 70% of TJA cases require a replacement surgery within a decade as a result of the accumulation of wear debris particles from an implant device, leading to particle-induced periprosthetic osteolysis [4–7].

Although, particle-induced osteolysis is significantly elevated in patients of an advanced age, most of the published pre-clinical studies were performed using experimental models of periprosthetic bone loss induced in young mice (less than three-months old) [8–10]. By contrast, using CSF1r-eGFP knock in (KI) mice, whose monocyte-lineage cells predominantly express enhanced green fluorescent protein (eGFP), we recently demonstrated that the molecular mechanisms of innate responses and bone osteolysis are significantly different between young (two-month old) and aged (twenty-four-month old) mice indicating the critical need to employ experimental models of particle-induced periprosthetic osteolysis involving aged mice [11,12].

While our understanding of the molecular mechanisms underlying wear debris-induced inflammation has substantially improved in recent decades [13,14] there is still a need for the development of novel therapeutic regimens for elevated periprosthetic osteolysis in senior individuals. Furthermore, a growing body of evidence suggests that the currently available antiresorptive therapies have serious side effects in relation to aging, such as the development of medication-related osteonecrosis of the jaw and may not improve bone quality or bone union ratios as expected [15–17]. Thus, a resurgence of interest has occurred in natural plant extracts as alternative sources of drugs for treating various bone diseases, including wear debris-induced osteolysis [17–20].

A triterpenoid saponin glycoside glycyrrhizin (GLY), an active component of licorice plant roots (Glycyrrhiza glabra), possesses multiple pharmacological properties, including anti-inflammation, anti-tumor, anti-aging, and anti-oxidative activities [21–23] It was also suggested that GLY attenuates tissue aging and promotes cell rejuvenation via the suppression of senescence-associated secretory phenotype (SASP) markers including macrophage migration inhibitory factor (MIF) and lysosomal cathepsins [24–26] Besides the role of GLY in the downregulation of SASPs expression, several studies have reported that it promotes the expression of senescence-protective nicotinamide adenine dinucleotide (NAD+) dependent deacetylases, known as sirtuins [27,28] It is true that the age-dependent accumulation of SASPs converts senescent cells into pro-inflammatory cells that alter osteoclastogenesis, leading to inflammatory osteolysis [11,29,30]. Furthermore, the incidence of senescent osteoclast precursors (OCPs) increases in the bone microenvironment of aging mice compared with young mice [31] and the inhibition of cellular senescence may prevent aging-related bone loss [32].

Although, GLY effectively inhibited osteoclast differentiation to prevent excessive bone resorption in vitro as well as in bone lesions induced in young mice [33–35], its therapeutic effects on the prevention of particle-induced inflammatory osteolysis in aged mice remain
unclear. Thus, in this study we aimed to address whether GLY mitigates inflammatory osteolysis as well as SASPs and promotes expression of senescence-protective sirtuins in an experimental model of particle-induced calvarial osteolysis using aged mice.

2. Material and methods

2.1. Animals

Aged (twenty-four-month-old) male CSF1r-eGFP-KI mice and their wild type strain C57BL/6 were used in this study. Animals were kept in a conventional room with a 12-hour light-dark cycle at constant temperature. Aged C57BL/6 animals were obtained as a donation from the NIA Aging Rodent Colony. The experimental procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at Nova Southeastern University.

2.2. RANKL-induced osteoclastogenesis and TRAP staining in vitro

Mononuclear bone marrow cells isolated from femur and tibia of aged wild type mice (twenty four month old) were seeded in 96-well plates at a density of $1 \times 10^5$ cells/well in α-MEM medium (Life Technologies) containing 10% FBS (Atlanta Biologicals) and 20 ng/ml of MCSF and 10 ng/ml of RANKL (BioLegend) recombinant proteins in the presence or absence of polymethylmethacrylate (PMMA) particles (mean diameter 6.5 μm; Bangs Laboratories), mouse MIF recombinant protein (R&D), and Glycyrrhizin (Tokyo Chemical Ind.) as described in earlier studies \[12,33,36,37\]. Five days later, cells were stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma). TRAP positive (TRAP+) cells with more than three nuclei were considered mature osteoclasts and were microscopically counted. The results were expressed as numbers per well.

2.3. Particle-induced calvarial osteolysis model

An experimental PMMA particle-induced osteolytic mouse model was used in this study. A suspension of PMMA particles in phosphate buffered saline (PBS) was prepared as we described earlier \[10\]. Then, the suspension of particles (150 μL/site) was injected subcutaneously over the calvarial bone by using a Tuberculin syringe (Covidien, Inc). In addition, a group of animals was additionally injected subcutaneously over the calvaria bone with several concentrations of glycyrrhizin solution in PBS (20 or 200 mg/kg) once every other day for ten days. Control animals received sham injection of PBS. The injection volume was 100 μL/mouse and the concentrations of GLY were determined based on the previously published observations \[38,39\].

2.4. In situ imaging of cathepsin B and K activities at calvarial bone lesions

At day 11, wild type mice were administrated intravenously (i.v.) with CatB 680 FAST™ and CatK 680 FAST™ (Perkin Elmer) fluorescent imaging probes according to the manufacturer’s instructions. Then, the fluorescence activities were measured from the PMMA particle-induced calvarial lesions by In-Vivo Xtreme II imaging system (Brucker) in anesthetized mice. The total flux (measured in photons per second) in the ROI were quantified using In-Vivo Xtreme Image Software (Brucker).
2.5. Isolation of total proteins from mouse calvariae

Mouse calvariae were collected from euthanized wild type animals and weighed. Then bones were mechanically homogenized on ice with a mortar and pestle in PBS with 0.05% Tween 20 in the presence of 10 μg/ml of ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Thermo Scientific). The suspension was centrifuged at 520 g and then collected proteins were isolated by ELISA.

2.6. ELISA

Concentrations of MIF and SDF-1 proteins from the collected cells supernatant and tissues suspension were measured using commercial sandwich ELISA kits (BioLegend).

2.7. Multicolor flow cytometry of OCPs

Mononuclear cells were isolated from calvarial tissues of CSF1r-eGFP-KI mice and then were incubated with anti-mouse CD16/32 (Fc-blocking antibody, Ab). Next, cells were stained with anti-CD11b conjugated to Pacific blue, anti-CD45 conjugated to Alexa 647, and anti-CXCR4 conjugated to PE Abs (BioLegend) to characterize OCPs as CD45+eGFP+CD11b+CXCR4+ cells. Cells were acquired on the BD FACSAria™ II Cell Sorter (BD Biosciences) and analyzed with FlowJo (ver. 10) software (Tree Star).

2.8. Histological analyses and immunohistochemistry

Dissected calvariae were decalcified in 10% EDTA (Thermo Fisher Scientific) and then were embedded in paraffin. Frontal calvarial Section 6 μm in thickness centered on the sagittal suture were obtained for histological analysis. To stain TRAP+ osteoclasts (OCs), sections were first incubated in 0.2 M acetate buffer containing 50 mM L-(+)-Tartaric acid (Sigma) at room temperature and then in TRAP staining solution (0.2 M acetate buffer, 50 mM L-(+)-Tartaric acid, 0.5 mg/ml Naphthol AS-MX phosphate, 1.1 mg/ml Fast Red ASTR salt; Sigma) at 37 °C. Finally, the sections were counterstained with hematoxylin solution (Sigma) at room temperature.

2.9. RNA Extraction and real-time PCR

Total RNA was isolated using PureLink™ RNA Mini Kit (Ambion, Life Technologies), following the manufacturer’s instructions, and subjected to reverse transcription with the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) in the presence of random primers and oligo-dT. Finally, sirtuin-1 (Mm01168521_m1), sirtuin-3 (Mm00452131_m1), and sirtuin-6 (Mm01149042_m1), cathepsin B (Mm00514443_g1), and cathepsin K (Mm00484039_m1) gene expressions were measured using TaqMan™ Fast Advanced PCR Master Mix (Applied Biosystems, Life Technologies). Data were analyzed using the 2^(-ΔΔCt) method normalized to GAPDH.

2.10. Statistical analysis

Data are displayed as mean ± SEM. Statistical significance was evaluated using a one-way ANOVA with post hoc Tukey’s test. A p < 0.05 was considered statistically significant. Data were analyzed using PAST 2.1 statistical software.
3. Results and discussion

3.1. Glycyrrhizin (GLY) regulates the production of pro-inflammatory MIF and homeostatic SDF-1/CXCL12 chemokines for OCPs in the particle-induced calvarial lesions of aged mice

While stromal cell-derived factor-1 (SDF-1)/CXCL12 produced in normal bone tissue was accepted as a chemoattractant factor to recruit circulating OCPs to the homeostatic bone remodeling site [40], our group previously reported that macrophage migration inhibitory factor (MIF), but not SDF-1, is engaged in the recruitment of OCPs to particle-induced osteolysis lesion in young mice, and that MIF suppresses the local production of SDF-1 [10]. Therefore, we first tested the effects of locally administrated GLY solution (Fig. 1A) on the expression of SDF-1 and MIF chemokines in the particle-induced peripheral osteolytic lesions of aged wild type mice using an ELISA assay. The concentration of MIF was significantly elevated in the PMMA particle-induced osteolytic lesions compared to the control group of mice (Fig. 1B, C). In contrast, the local administration of GLY significantly reduced the MIF concentration in calvaria lesions compared to mice treated with a sham control (Fig. 1B). We also detected that SDF-1 levels in the PMMA-induced calvaria lesions of the GLY-treated mice were significantly elevated compared to the mice treated with PMMA alone (Fig. 1C); these findings correspond with the findings of our previous report, which showed that the downregulation of MIF has a positive effect on SDF-1 levels in PMMA-induced calvaria osteolysis lesions using CSF1r-eGFP-KI mice [10].

In addition to MIF and SDF-1 chemokines, it was demonstrated that MCP-1 (CCL-2) chemokine promotes macrophage recruitment to particle-induced bone lesions [41–43]; however, the effects of MCP-1 on the macrophage chemotaxis were significantly mitigated in the MIF--knock out (KO) mice, indicating that MIF plays an essential role in the recruitment of OCPs to bone lesions [44,45]. Furthermore, a large number of studies demonstrated that MIF-KO mice have an extended lifespan and experience reduced osteoarthritis and particle-induced osteolysis severity compared to wild-type control mice, suggesting the important role of MIF as an SASP marker in the aging process and bone osteolysis [12,46–48]. Since aging decreases the level of homeostatic SDF-1 in peripheral blood while up-regulating the expression of pro-inflammatory MIF in various pathologies associated with aging, including peripheral osteolytic lesions [46,49–53], it is plausible that GLY could contribute to the prevention of osteolytic lesions in aged mice via the suppression of pro-inflammatory MIF production, restoring the concentration of homeostatic SDF-1.

3.2. GLY suppresses recruitment of CXCR4+OCPs to particle-induced osteolytic lesions in aged mice

Growing evidence suggests that bone inflammation promotes chemokine-induced recruitment of OCPs from the blood stream to bone lytic lesions, leading to elevated osteoclastogenesis at the site of inflammation [54,55]. Our previously published observations indicated that ligation of MIF and SDF-1 with CXCR4 receptor promotes OCPs chemotaxis and osteoclastogenesis in vitro as well as in the experimental model of particle-induced osteolysis using young CSF1r-eGFP-KI mice [10]. Because CSF1r-eGFP-
KI mice express eGFP protein in osteoclast precursors, it was suggested that this transgenic strain could be used to elucidate the molecular mechanisms underlying chemotaxis of OCPs to osteolytic lesions in relation to aging [11,56]. Consequently, we examined next whether local administration of GLY could affect the recruitment of CXCR4+OCPs to PMMA particle-induced osteolysis in aged CSF1r-eGFP-KI mice by multicolor flow cytometry assay. It was clearly demonstrated that PMMA particles significantly increased the infiltration of CXCR4+OCPs in the calvarial lesions compared to the healthy group of aged mice. In contrast, a local injection of GLY significantly suppressed the infiltration of CXCR4+OCPs to the particle-induced bone lesion compared to the group of mice that received the control sham injection (Fig. 2A–C). These data concur with the findings of a previous study, which indicated that GLY suppresses the CXCR4-dependent migration of monocytes and fibroblasts in vitro and in vivo [57]. It is notable that a number of studies have detected an elevated expression of CXCR4 mRNA in senior patients when compared with younger individuals [58–61]. Altogether, our results strongly suggest that locally administered GLY diminished the migration of CXCR4+OCPs to particle-induced osteolytic lesions induced in aged mice.

3.3. GLY diminishes the effect of particles on the size of osteolytic calvarial lesions in aged mice

The elevated migration of CXCR4+OCPs to particle-induced osteolytic lesions was positively correlated with upregulated osteoclastogenesis and bone loss around the implant device [62–65]. Since GLY suppresses the recruitment of OCPs to the particle-induced calvaria osteolysis (Fig. 2), we evaluated the impact of GLY on PMMA particles- and recombinant MIF-elicited osteoclastogenesis in vitro using RANKL-primed bone marrow derived macrophages (BMDM) isolated from aged wild type mice. Addition of PMMA particles and recombinant MIF to RANKL-stimulated BMDM cells dramatically enhanced the development of TRAP+ multinucleated osteoclasts (OCs) (Fig. 3A, B). In contrast, GLY significantly reduced the number of OCs exposed to PMMA and MIF in vitro (Fig. 3A, B).

To further confirm our in vitro findings, we examined the effects of GLY on the expression of some important markers for osteoclastogenesis, including De-stamp, Oc-stamp, and Acp5/Trap mRNAs, and the development of osteolytic lesions along with numbers of TRAP+ cells in the PMMA particle injection site at the calvaria of aged mice. In response to a PMMA particles injection followed by GLY local treatment, the expression patterns of De-stamp, Oc-stamp, and Acp5 were significantly reduced (Fig. 4A). Furthermore, the size of osteolytic lesions and the number of TRAP+ osteoclasts in the mouse calvaria that received PMMA particles were significantly larger than those found in mice with particle-induced osteolytic lesions treated with GLY (Fig. 4B, C). These results accorded with those of previous reports, showing that GLY effectively inhibits RANKL-induced osteoclastogenesis in vitro and in an experimental mouse model of osteoporosis [33,37]. However, no effect of orally-administrated GLY on bone properties in ovariectomized rats was detected [66]. A previous study reported that the bioavailability of oral administering GLY is low because of its poor absorption in the intestine, indicating that an intraperitoneal administration of GLY significantly improves its beneficial effect on bone health in a model of osteoporosis using young mice [37]. These findings indicate that locally injected GLY attenuated the expression
of pro-osteoclastogenic markers and reduced the amount of bone loss and the number of TRAP+ osteoclasts in vitro as well as in the osteolytic calvarial lesions induced in aged mice.

3.4. GLY reduces activities of bone resorptive cathepsins in the calvarium lesions of aged mice induced by PMMA particles

It is well-established that the activity of lysosomal cathepsin proteases is significantly elevated in senescent cells compared with young cells, indicating that cathepsins are a conserved component of SASPs [67–69]. Among the eleven known cathepsins, cathepsin B plays an essential role in the RANKL-mediated fusion of mononucleated OCPs, while cathepsin K is secreted by activated multinucleated osteoclasts to degrade various matrix proteins during bone resorption [70–72]. We examined whether GLY downregulates activities of cathepsins B and K in the particle-induced osteolytic lesions of aged wild type mice using real-time PCR and in situ intravital imaging assays. According to the real-time PCR evaluation, expression patterns of cathepsins B (catB) and K (catK) were significantly elevated in the PMMA-particle-induced osteolysis compared with control mice (Fig 5A, D), partially resembling findings of previously published reports, which found that patients with periprosthetic osteolysis have an elevated expression of cathepsin K [73,74]. In addition, some studies have reported that the levels of RANKL-induced osteoclastogenesis and pathogenic bone loss are positively correlated with elevated activities of cathepsins B and K in OCPs in relation to aging [68,75–77]. In this study, local injection of GLY significantly attenuated expression patterns of cathepsins B and K genes in the osteolytic lesions induced in aged mice (Fig. 5A, D). Furthermore, GLY significantly reduced the signal intensities for both cathepsins B (Fig. 5B, C) and cathepsin K (Fig. 5E, F) at the calvaria PMMA-induced inflammation site, indicating that GLY effectively downmodulated the enzymatic activities of cathepsins B and K in the bone lesions of aged mice. Since selective pharmaceutical inhibition of cathepsin B and K activities significantly reduces bone resorption in vitro as well as in vivo [78], these results strongly suggest that locally-administered GLY mitigates the activities of cathepsins B and K in the aging experimental model of particle-induced calvarial osteolysis.

3.5. GLY diminishes the negative effect of PMMA particles on the expression of senescence-protective sirtuins in calvarial lesions of aged mice

Some studies reported that lysosomal cathepsins B and K promote the degradation of ubiquitously-expressed senescence-protecting sirtuins, which may also suppress particle-induced osteolysis [79,80]. Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that are implicated in a plethora of biological processes including metabolism, stress responses, and tumorigenesis [81]. Among the seven-known mammalian sirtuin families, growing evidence shows that sirtuin-1, sirtuin-3, and sirtuin-6 are essential factors in delaying cellular senescence and extending organismal lifespan by reversing some aspects of aging [82,83]. Sirtuin-1 and sirtuin-6 are predominately found in the nucleus, while sirtuin-3 resides in the mitochondria [83]. Nevertheless, the GLY-dependent effects on expression patterns of sirtuin-1, sirtuin-3, and sirtuin-6 in the particle-induced osteolytic lesions remains limited. As GLY suppressed the development of osteolytic lesion and reduced the activity of cathepsins B and K (Figs. 3–5), we next evaluated the effect of GLY
on the expression patterns of sirtuin-1, sirtuin-3, and sirtuin-6 genes on osteolytic lesions of aged wild type mice. It was observed that the levels of sirtuin-1 and sirtuin-6 mRNAs in the group of aged mice locally injected with PMMA/GLY were significantly higher compared to the group treated with PMMA alone (Fig. 6A, C). It is important to note that, sirtuin-1 also enhances the expression of sirtuin-6 by interacting with FOXO3a and NRF1 at the sirtuin-6 promoter [84]. We also detected that GLY had either no or little significant effect on the expression of mitochondrial-localized sirtuin-3 in the peripheral osteolytic lesions induced in aged mice (Fig. 6B), indicating that GLY mainly promotes the expression of nuclear-localized sirtuin-1 and sirtuin-6. A recently published study demonstrated that sirtuin-3 elevated RANKL-mediated osteoclastogenesis in aged male mice, suggesting that sirtuin-3 promotes aging-related bone loss [85]. Our results support the previous finding by Deng et al. [86], that sirtuin-1 expression was significantly attenuated in the biopsies collected from patients with particle-induced osteolysis. Furthermore, it has been also demonstrated that the activation of sirtuin-1 and sirtuin-6 suppresses aging-dependent osteoclastogenesis [87,88]. Taken together, these results strongly suggest that GLY elevates the expression of senescence-protecting sirtuin-1 and sirtuin-6 in the PMMA particles-induced calvarial lesions, supporting our key finding that GLY promotes tissue rejuvenation and reduces bone osteolysis in aged mice.

4. Conclusion

In this study, we investigated the effects of locally injected glycyrrhizin (GLY) on experimental calvarial osteolysis induced by PMMA particles in aged (twenty-four-month-old) mice. We demonstrated that GLY significantly diminished the size of inflammatory osteolysis in aged mice via the number of CXCR4+OCPs and TRAP+ osteoclasts. Our results also indicated that GLY significantly downmodulates the amount of SASPs, including pro-inflammatory MIF chemokine and cathepsins B and K in the bone lesions of aged mice. We also demonstrated that GLY elevated the amount of homeostatic SDF-1 chemokine and the expression of senescence-protective factors, such as sirtuin-1 and sirtuin-6, in the calvarial lesions of aged mice. Therefore, GLY can be used for the development of novel therapies to control particle-induced osteolytic lesions and SASPs in senior patients after TJA.

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Fig. 1.
Glycyrrhizin (A; chemical structure) regulates the production of pro-inflammatory MIF (B) and homeostatic SDF-1/CXCL12 (C) chemokines for OCPs in the particle-induced calvarial lesions of aged mice. Calvarial tissues were sampled from wild type aged (twenty-four-month-old) mice and then were evaluated by an ELISA assay. *p < 0.05, **p < 0.01; ***p < 0.001. GLY – Glycyrrhizin.
Fig. 2.
The impact of Glycyrrhizin (GLY) on the incidence of CXCR4+ osteoclast precursors (OCPs) in PMMA particle-induced calvarial lesions of aged (twenty-four-month-old) CSF1r-eGFRP-knock in mice. Representative contour plots (A) and percentage of eGFP+CD11b+ OCPs (B) identified in osteolytic lesions induced in CSF1r-eGFP-knock in mice that received subcutaneously over the calvarial bone: Control (no particles), PMMA particles alone, and PMMA particles + GLY solution. C: expression of CXCR4+ receptor on the surface of OCPs detected in the osteolytic lesions after treatment with GLY. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 3.
The effects of Glycyrrhizin (GLY) solution on aged RANKL-primed osteoclast precursors exposed to PMMA particles and recombinant MIF protein in vitro. Microscopic evaluation of the TRAP staining and number of TRAP multinucleated osteoclasts per well formed from the RANKL-stimulated bone marrow derived macrophages exposed to PMMA particles (A, B) and MIF (C, D) in the presence of GLY. Bone marrow derived macrophages were collected from twenty-four-month-old C57BL/6 mice. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 4.
Glycyrrhizin (GLY) diminishes effects of PMMA particles on the size of osteolytic calvarial lesions in aged mice. (A) Expression of osteoclastogenesis-related Oc-stamp, Dc-stamp, and Acp 5/Trap genes in the dissected calvarial tissues of control (no particle placed) and PMMA particle-induced bone lesions of sham and GLY-treated aged wild type (twenty-four-month-old) mice. μ-CT images and histological evaluation of TRAP osteoclasts (B and C) in the PMMA particle-induced calvarial lesions of aged mice that received: Control (no particles), PMMA particles alone, and PMMA/GLY injection. D: The number of TRAP+ osteoclasts per 0.1 mm².
osteoclasts measured in a microscopic field (0.01 mm$^2$) of TRAP-stained sections. Because no significant effect of GLY at 20 mg/kg concentration was observed, only a group of mice treated with 200 mg/kg of GLY solution was evaluated by μ-CT and immunohistochemistry. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 
Fig. 5. Glycyrrhizin (GLY) reduces activities of bone resorptive cathepsin B and K in the calvarium lesions of aged mice induced by PMMA particles. Expression patterns of cathepsin B and K mRNA (A, D) and in situ molecular imaging of cathepsin B and K activities (B, E) in the PMMA particle-induced calvarial lesions of wild type aged (twenty-four-month-old) mice that received: Control (no particles), PMMA particles alone, and PMMA/GLY injection. The levels of signal intensity of cathepsin B (C) and cathepsin K (F) with respect to total flux quantified from the molecular imaging assay. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 6.
Glycyrrhizin (GLY) diminishes negative effects of PMMA particles on the expression of senescence-protective sirtuins (Sirt) in bone lesions of aged mice. Sirt-1 (A), sirt-3 (B), and sirt-6 (C) gene expression levels in the PMMA particle-induced calvarial lesions of aged mice that received: Control (no particles), PMMA particles alone, and PMMA/GLY injection. *p < 0.05, **p < 0.01.