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Dexamethasone Controlled Release on TGF-α1 Treated Vocal Fold Fibroblasts

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Abstract

Objective—Corticosteroids may be beneficial in treating vocal fold scarring. Current drug delivery methods do not permit controlled corticosteroid release. Here we investigate the effects of poly-lactic-co-glycolic acid (PLGA) microparticles loaded with the corticosteroid dexamethasone in reducing collagen synthesis and inflammation in vocal fold fibroblasts treated with and without TGF-β1.

Study Design—Experimental, in vitro study.

Methods—PLGA microparticles of differing molecular weight and terminating moieties were synthesized using a hydrogel template method. The release of dexamethasone was characterized from these microparticles over 4 days. Based on the release studies, ester-terminated low molecular weight PLGA microparticles were loaded with dexamethasone and applied to TGF-β1 treated vocal fold fibroblasts for 4 days. Quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assays (ELISAs) were used to assess the effects of released dexamethasone on collagen synthesis and inflammatory mediators.

Results—COL3A1 and COL1A2 were significantly down-regulated after exposure to ester-terminated low molecular weight PLGA microparticles loaded with dexamethasone. The loaded microparticles also reduced interleukin-6 synthesis.

Conclusion—These data show promise in using a PLGA microparticle-based delivery system to control dexamethasone release over 4 days. Our findings lay the groundwork for developing more effective treatments for vocal fold scarring.

Keywords

dexamethasone; drug delivery; PLGA microparticles; vocal fold scar

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Declaration of Conflicting Interests

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Introduction

Vocal fold scarring is characterized by a disorganized and stiff extracellular matrix with significant increases in collagen gene expression.[1–3] Scarring results from vocal fold injury and changes the biomechanical properties of the vocal folds,[3] which can result in dysphonias.[2] Effective treatments for vocal fold scarring remain elusive. One common treatment involves augmenting the vocal folds with injectable materials.[4,5] However, current injectable materials degrade and only offer a temporary solution.[1] Here we investigate a drug delivery method that would facilitate controlled release of a therapeutic to better treat vocal fold scar.

Dexamethasone was selected as the therapeutic for 2 reasons. First, it is a highly effective anti-inflammatory agent.[6–8] Second, it plays an anti-fibrotic role. In vitro studies have shown that dexamethasone decreases glucocorticoid receptor expression, fibroblast proliferation, collagen synthesis induced by TGF-β1, and enzymes related to extracellular matrix turnover.[7] In vivo studies have demonstrated that dexamethasone decreases collagen deposition during the acute healing phase in surgically wounded rabbit vocal folds.[9] Dexamethasone is typically administered to the vocal folds via injection. However, the full potential of dexamethasone has not been achieved through injection. This could be related to poor retention of drug at the injection site. Although drug efficacy could be improved with multiple dexamethasone injections, patient safety and tolerance quickly become significant barriers in pursuing such a rigorous treatment regimen. Therefore, alternative methods for sustaining target drug levels are needed.

Here we demonstrate the effectiveness of slow (4 day) release of dexamethasone in reducing collagen expression and inflammation in TGF-β1 treated immortalized human vocal fold fibroblasts. TGF-β1 is implicated in vocal fold scarring. To facilitate controlled drug delivery, we developed a polylactic-co-glycolic acid (PLGA) microparticle delivery system, which was loaded with dexamethasone. PLGA is an aliphatic polyester[10] whose rate of degradation can be controlled.[11–15] Upon degradation, lactic and glycolic acids are formed, which are biocompatible as they can be broken down in the tricarboxylic acid cycle and be reused by the body or expelled.[12] PLGA has been used to deliver a variety of therapeutics, including steroids, peptides, and growth factors,[11–16] and was therefore selected as the platform for delivering dexamethasone. PLGA is also approved for use in humans by the US Food and Drug Administration.[11–14] Furthermore, PLGA particle formation and drug loading schemes are well characterized.[12–14] To guarantee the controlled delivery of an effective therapeutic dose through direct tissue injection, we synthesized micron sized PLGA particles. The micron size allows for more therapeutic to be loaded and subsequently released. PLGA microparticles were synthesized using a hydrogel template based method.[17] This technique allows for the production of homogenous microparticles and more uniform drug release profiles than other production techniques.[18]

The in vitro studies described here used an immortalized human vocal fold fibroblast cell line.[19] Fibroblasts play an important role in regenerating scarred vocal fold lamina propria by facilitating the deposition of many important extracellular matrix components, including collagen, elastin, and hyaluronic acid.[1] Verifying the slow release of dexamethasone and its

effectiveness in reducing TGF-β1 induced collagen synthesis and inflammation in fibroblasts was accomplished in the following manner. Dexamethasone release studies were completed for 2 different PLGA microparticles. Based on the release kinetics, optimal PLGA microparticles were selected, loaded with dexamethasone, and applied to TGF-β1 treated fibroblasts. Quantitative polymerase chain reaction (qPCR) was used to assess collagen gene expression. Enzyme-linked immunosorbent assays (ELISAs) were used to assess levels of interferon-gamma (IFN-γ), interleukin-1beta (IL-1β), interleukin-6 (IL-6), and transforming necrosis factor-alpha (TNF-α). These inflammatory mediators are released by fibroblasts.[20–23] Data on the effectiveness of PLGA microparticles loaded with dexamethasone in reducing collagen synthesis and inflammation will lay the groundwork for developing long-term treatments for vocal fold scarring.

Materials and Methods

Microparticle Fabrication

Microparticles were synthesized using a hydrogel template method.[17] Briefly, a polydimethylsiloxane (Dow Corning, Midland, Michigan, USA) template was generated from a prefabricated silicon wafer (50 um × 30 um ; Akina, Inc., West Lafayette, Indiana, USA). The patterned side of the template was covered in a thin film of melted (50°C–55°C) porcine gelatin (30% w/v; Sigma-Aldrich, St. Louis, Missouri, USA) and allowed to cool at 4°C for 5 minutes. Post cooling, the gelatin solidified and was gently peeled away, resulting in the formation of a deep welled gelatin template. Wells of the gelatin template were filled with a 9:1 dichloromethane:methanol solution (Sigma-Aldrich) in which 1% w/v dexamethasone (Alfa-Aesar, Ward Hill, Massachusetts, USA) was combined with either 1 of the following: (1) 40% w/v low molecular weight (6700 Da) ester-terminated PLGA or (2) 10% w/v high molecular weight (~111 500 Da) ester-terminated PLGA (Lactel, Birmingham, Alabama, USA). The organic solvents were allowed to evaporate for 5 to 10 minutes at room temperature. The filled gelatin templates were then dissolved using 60°C deionized water, releasing the dexamethasone loaded PLGA microparticles into solution. Microparticles were dried for 12 to 24 hours in a lyophilizer and stored at −20°C for later use.

Dexamethasone Release Study

Two milligrams of each type of PLGA microparticle with or without dexamethasone were weighed out into tubes. One hundred uL of neutral, phosphate-buffered saline (PBS) was added, and tubes were placed on an orbital shaker at 400 rpm at 37°C. At various time intervals, the microparticles were pelleted at 4000 g for 5 minutes. The supernatant was removed. The pelleted microparticles were resuspended in 100 uL of fresh, neutral PBS. Finally, the tubes were put back in the orbital shaker, as described previously, and the procedure was repeated at the next time point.

Percent cumulative dexamethasone release was determined by comparing the amount of dexamethasone released from each PLGA microparticle type over time to the total amount of dexamethasone loaded originally into 2 mg of that corresponding PLGA microparticle type. Dexamethasone released and total dexamethasone were quantified by measuring the

absorbance at 240 nm (SpectraMax M5 spectrophotometer; Molecular Devices, Sunnyvale, California, USA), the peak absorbance of dexamethasone, and comparing the absorbance to that generated by a dexamethasone standard curve. For total dexamethasone, a fresh 2 mg of the different PLGA microparticles were dissolved in a 9:1 solution of dichloromethane to methanol before being read on the spectrophotometer. Total loading showed that 0.007 mg and 0.0596 mg of dexamethasone were loaded in 2 mg of ester-terminated low molecular weight and high molecular weight PLGA microparticles, respectively.

Cell Culture

The immortalized human vocal fold fibroblast cell line was obtained from Dr Susan Thibeault from the University of Wisconsin, Madison.[19] Chemicals were obtained from Sigma-Aldrich unless specified otherwise. The fibroblasts were grown in Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% MEM non-essential amino acids, and 200 ug geneticin (G418; Teknova, Hollister, California, USA) per mL of media. The fibroblasts were initially seeded at 2000 cells/well in a 96-well plate and left to attach overnight at 37°C and 5% CO_2. The cells (barring the control group) were incubated in media described previously that also contained 10 ng/ml TGF-β1, minus fetal bovine serum (Biosource, Camarillo, California, USA) for 48 hours at 37°C and 5% CO_2. Next, the cells were treated with (1) media alone, (2) media and 50 ng/mL free dexamethasone, (3) media and 0.01 mg/well dexamethasone loaded PLGA microparticles, and (4) media and 0.01 mg/well PLGA only microparticles for 24 hours at 37°C and 5% CO_2. The cells were then spun at 2000 g for 10 minutes to remove the microparticles from suspension in the media as a way to mimic in vivo entrapment in tissue. The supernatants from each well were removed to mimic the clearance of drug as seen in in vivo systems, and fresh or TGF-β1 media were added appropriately. Finally, the fibroblasts were left to grow at 37°C and 5% CO_2 for 72 hours. Fibroblasts reached confluence in 4 days. Therefore, upon completion of 4 days of treatments, the media was collected and stored at −80°C for further analysis. The RNA was immediately purified from the cells as described in the following.

qPCR

The RNA was purified from the fibroblasts using a Nucleospin total RNA isolation kit (Clontech, Mountain View, California, USA). Once purified, the RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Grand Island, New York, USA). The qPCR reactions were conducted using TaqMan gene expression master mix (Life Technologies) combined with gene-specific probes (Life Technologies) for β-actin, COL1A2, COL3A1, and template cDNA from the different samples. Four replicates were used. These reactions were run under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (Applied Biosystems 7500, Life Technologies). The endogenous control was human β-actin. Average cycle threshold (Ct) from each sample was used for the calculation of gene expression levels using the relative comparative Ct method.

ELISA

The human pro-inflammatory cytokines IFN-γ, IL-1β, IL-6, and TNF-α were analyzed utilizing a sandwich ELISA (Meso Scale Discovery, Rockville, Maryland, USA). Three replicates were used. The analysis was conducted according to manufacturer instructions (MSD Sector Imager 2400, Meso Scale Discovery).

Statistical Analysis

Data were summarized as means ± SD. A 1-way ANOVA with Tukey post hoc testing was utilized for statistical analysis. The alpha level was set to 0.05. All statistical analyses were computed using Minitab statistical software (Minitab Inc, State College, Pennsylvania, USA).

Results

Dexamethasone Release Study

The hydrogel template based method successfully produced dexamethasone loaded microparticles.[17] Relatively uniform PLGA microparticles with an anticipated diameter of 50 μM were obtained (Figure 1). A 4-day study was conducted to ascertain the release profiles of dexamethasone from the 2 types of PLGA microparticles (Figure 2). Four days was the desired length of time because the fibroblasts became confluent beyond this time period. An initial ~12-hour burst release of dexamethasone was observed for both PLGA microparticles. After the 12-hour period, release profiles diverged. The ester-terminated low molecular weight PLGA microparticles released maximum dexamethasone around 4 days. The ester-terminated high molecular weight PLGA microparticles released dexamethasone at a much slower rate and were deemed to be therapeutically ineffective for the 4-day timeline selected.

TGF-β1 Mediated Collagen Synthesis

Exposure to exogenous TGF-β1 induced a cell phenotype characterized by a significant increase in collagen gene expression (Figures 3A and 3B). Four days of exposure to TGF-β1 caused a 2.75- ± 0.05-fold increase in COL1A2 and a 3.5- ± 0.05-fold increase in COL3A1. Both free dexamethasone and ester-terminated low molecular weight PLGA microparticles loaded with dexamethasone reduced COL1A2 and COL3A1 in TGF-β1 treated fibroblasts after 2 days (data not shown). However, by the end of the study at day 4, free dexamethasone no longer maintained its anti-fibrotic efficacy as COL1A2 and COL3A1 expression returned to normal TGF-β1 induced levels (Figures 3A and 3B). In contrast, ester-terminated low molecular weight PLGA microparticles loaded with dexamethasone maintained anti-fibrotic efficacy by continuing to significantly depress COL1A2 and COL3A1 expression levels at 4 days ($P < .05$) (Figures 3A and 3B). PLGA-only microparticles also elicited anti-fibrotic behavior with a significant reduction in COL3A1 but not COL1A2 expression at 4 days ($P < .05$) (Figures 3A and 3B).

Pro-inflammatory Mediators

Control fibroblasts released the following levels: 113.8 ± 32.6 pg/mL IL-6 (Figure 4), 0.5 ± 0.2 pg/mL IL-1β, 16.6 ± 5.2 pg/mL IFN-γ, and 1.9 ± 1.0 pg/mL TNF-α. Levels for IL-6 fell significantly after exposure to TGF-β1 to 43.9 ± 1.0 pg/mL ($P < .05$) (Figure 4). Levels also decreased for the other cytokines: 0 ± 0 pg/mL IL-1β, 1.9 ± 3.2 pg/mL IFN-γ, and 0 ± 0 pg/mL TNF-α after exposure to TGF-β1 (data not shown). Both free dexamethasone and ester-terminated low molecular weight PLGA microparticles loaded with dexamethasone significantly reduced IL-6 levels, with the greatest decrease observed for the ester-terminated low molecular weight PLGA microparticles (Figure 4). However, dexamethasone treatments had no significant impact on further reducing IL-1β, IFN-γ, or TNF-α.

Discussion

Current treatments for vocal fold scarring are inadequate. Injection of bulking materials only yields a transient beneficial effect due to rapid degradation.[1] Dexamethasone offers a promising treatment[7–9] through direct injection, but success is minimized because of short residence times. We sought to develop a means to increase the effectiveness of dexamethasone through the use of poly-lactic-co-glycolic acid (PLGA) microparticles. We hypothesized that PLGA microparticles would serve as depots for slower administration of dexamethasone. We synthesized 2 types of PLGA microparticles that varied in molecular weight and terminating moieties and compared dexamethasone release. We selected ester-terminated low molecular weight PLGA microparticles because the release of dexamethasone from these microparticles was gradual over 4 days. The 4-day time point was selected as cells reached confluence at 4 days. Future studies should include longer time points to better replicate clinical situations.

Dexamethasone released from the ester-terminated low molecular weight PLGA microparticles significantly decreased both COL1A2 and COL3A1 gene expression. These transcript levels are normally up-regulated in the presence of TGF-β1.[3,24,25] We confirmed the up-regulation of these genes in the presence of TGF-β1 and demonstrated a significant decrease following dexamethasone treatment. We also investigated changes in protein expression. Decreases in COL1A2 and COL3A1 gene expression were not accompanied by changes in protein expression (data not shown). Possible reasons for the nonsignificant changes in protein expression include probing for a specific type of collagen rather than total collagen, as described previously.[7] We chose to look at 2 individual types of collagen as they have been shown to be the most prevalent types of collagen present within the vocal folds.[26] In addition, multiple studies have reported on the importance of these collagen types in scarring.[27–29] It is also possible that changes in protein expression and extracellular remodeling take longer to manifest than changes in gene expression. It is noteworthy that the PLGA microparticles alone also reduced collagen synthesis, as documented by a significant decrease in COL3A1 but not COL1A2 expression. The reasons for these findings are unclear and will be the subject of future investigation.

TGF-β1, a cytokine itself,[30] acted as a pro-fibrotic agent by up-regulating collagen expression in fibroblasts. However, in the context of inflammation, TGF-β1 acted as an anti-inflammatory agent, significantly decreasing the secretion of pro-inflammatory

cytokines.[30,31] Understanding the role that both TGF-β1 and dexamethasone have on inflammation is critical as inflammation contributes to the formation of scar tissue in the vocal folds and other parts of the body.[22,32–34] The anti-inflammatory effects of dexamethasone were demonstrated here in decreased protein expression levels for IL-6 beyond that seen with TGF-β1. Both free dexamethasone and ester-terminated PLGA microparticles loaded with dexamethasone appeared equally effective in reducing IL-6 synthesis. IL-6 increases collagen production and plays a role in scarring.[35–37] However, dexamethasone was not effective in suppressing production of the other pro-inflammatory cytokines probed in this study beyond that obtained with TGF-β1.

The advantages of PLGA microparticles described here include the ability to control the release profile of dexamethasone, and most small molecule hydrophobic therapeutics, simply by varying the molecular weight and/or terminal moiety of the PLGA.[38] Furthermore, physical particle size and shape can also be manipulated as yet another means by which therapeutic release can be manipulated.[17,18] These advantages of PLGA microparticles fill a gap in drug delivery systems being developed for treatment of vocal fold scarring. Currently, research has focused on 2 routes of therapeutic delivery in the vocal folds. The first is via nanoparticle based delivery systems.[39] Due to their small size, these systems have many desirable attributes, including effective intercalation into the tissue and relatively quick release of loaded therapeutics due to high surface to volume ratios. As a result, they could prove well suited in treating vocal fold disorders that need short-term administration of therapeutic. But these delivery systems are limited in addressing more long-term problems like reverting an established vocal fold scar or tumor. The second major therapeutic delivery route is through the use of hydrogels loaded with therapeutics.[40,41] The advantages of this type of system is that the hydrogel can serve as a bulking agent that provides structural support and pliability to damaged tissue while releasing therapeutic to facilitate proper healing. However, therapeutic release from these hydrogel systems is potentially very quick as these systems are designed to readily absorb water, which once present can allow for quick diffusion of any loaded therapeutic out of the hydrogel. Conversely, the ester-terminated low molecular weight PLGA microparticles loaded with dexamethasone described here provide a third type of therapeutic delivery that shows promise in overcoming the disadvantages of nanoparticle and hydrogel based systems. By increasing particle size from the nanoscale to the microscale, PLGA microparticles provide the ability to load more therapeutic. Simultaneously, PLGA microparticles reduce the surface to volume ratio, helping to decrease the rate of therapeutic release and particle degradation. This feature in combination with the material properties of PLGA result in a drug delivery system that can facilitate sustained release of biologically relevant dosages of loaded therapeutics. These data therefore lay the foundation for developing effective treatments for a myriad of vocal fold conditions.

Conclusions

Here we present data on the effect of 4-day release of dexamethasone in reducing TGF-β1 mediated collagen synthesis and inflammation. Ester-terminated low molecular weight PLGA microparticles were loaded with dexamethasone. These microparticles effectively released dexamethasone over 4 days and down-regulated COL1A2 and COL3A1 expression

and synthesis of IL-6 cytokine. Our future work will look at using this microparticle based delivery system to control the delivery of other important therapeutic agents to the vocal folds.

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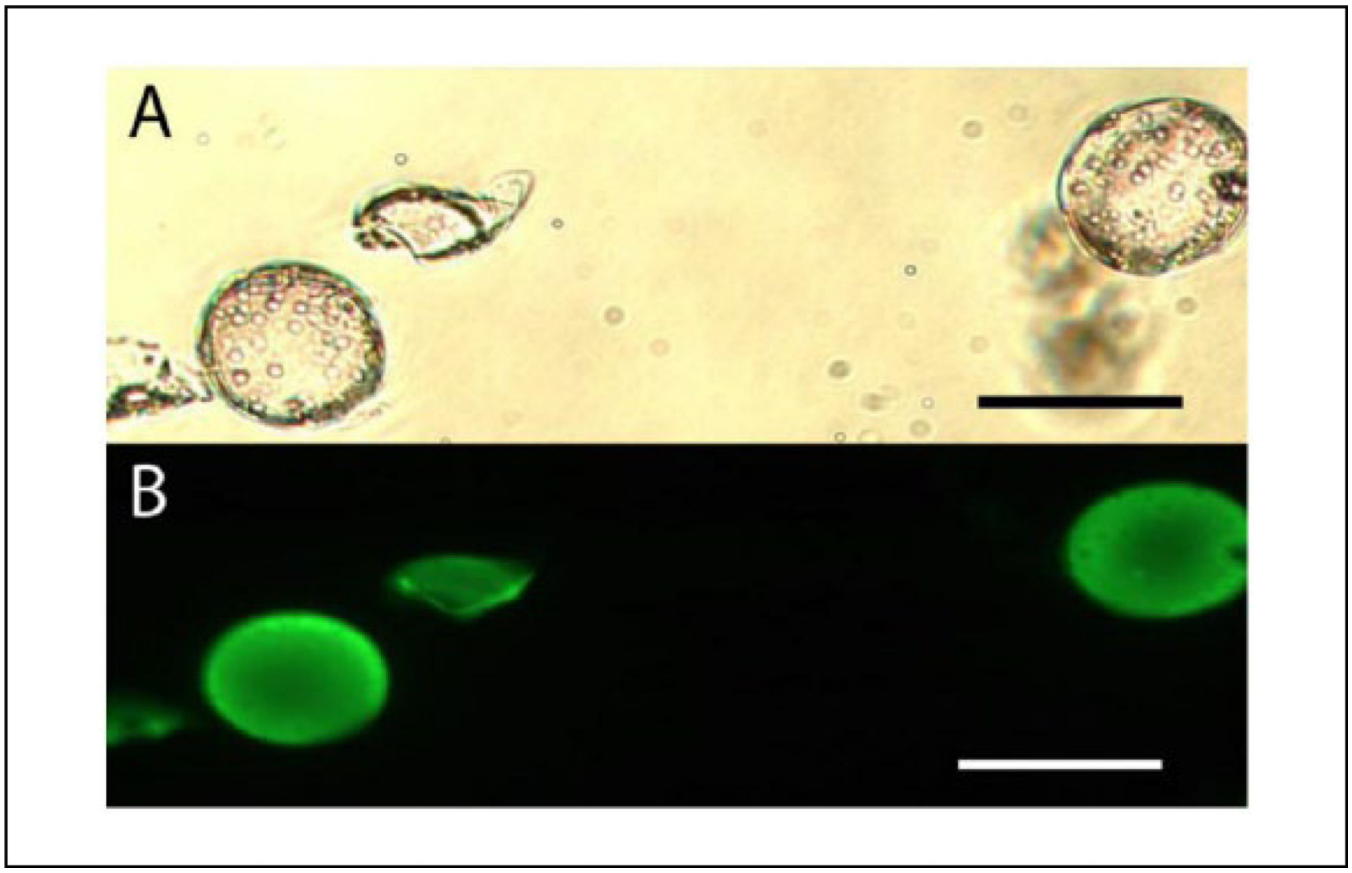

Figure 1.
Ester-terminated low molecular weight poly-lactic-co-glycolic acid (PLGA) microparticles. (A) Brightfield image and (B) fluorescent image. Scale bars = 50 um.

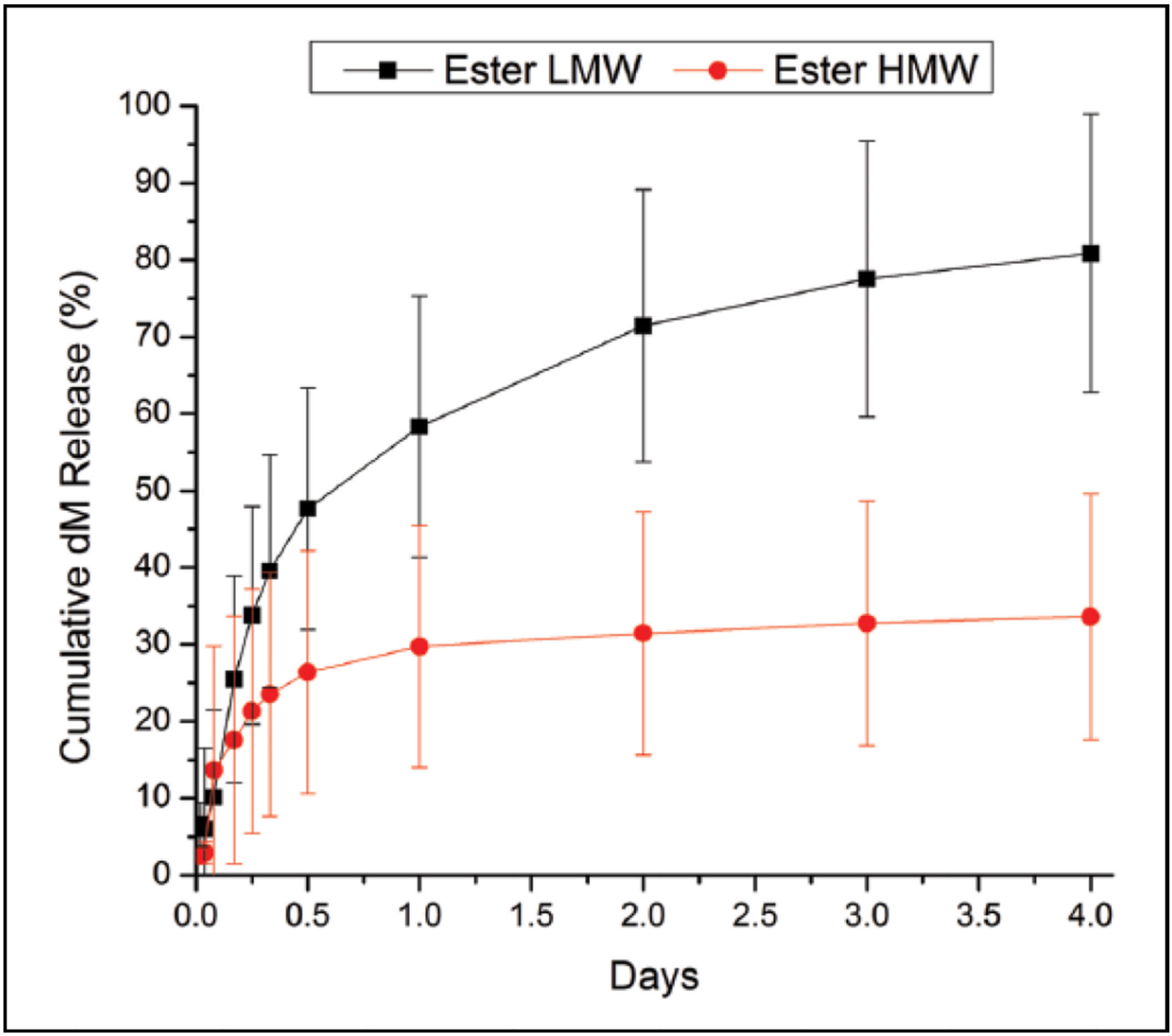

Figure 2.

Comparative release profiles of percentage cumulative dexamethasone (dM) over 4 days from ester-terminated low molecular weight (LMW) and ester-terminated high molecular weight (HMW) poly-lactic-co-glycolic acid (PLGA) microparticles.

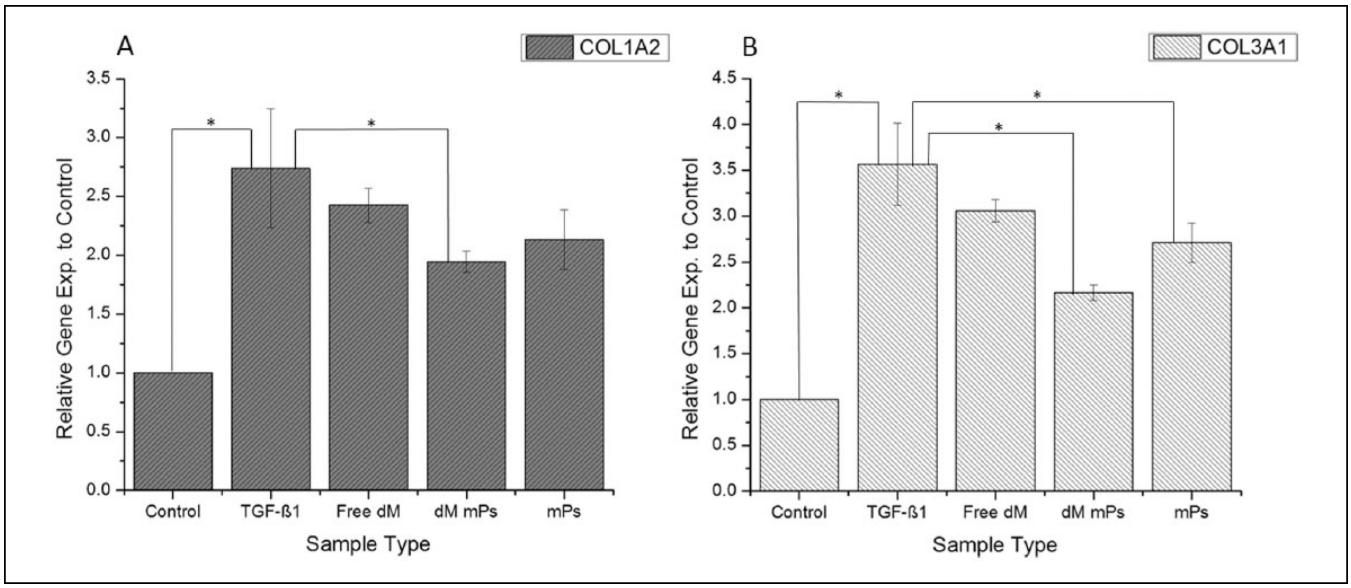

Figure. 3.

TGF-β1 treatment significantly increased (A) COL1A2 and (B) COL3A1 gene expression compared to control. Ester-terminated low molecular weight poly-lactic-co-glycolic acid (PLGA) microparticles loaded with dexamethasone (dM mPs) significantly down-regulated (A) COL1A2 and (B) COL3A1 gene expression compared to TGF-β1 treatment. *$P < .05$.

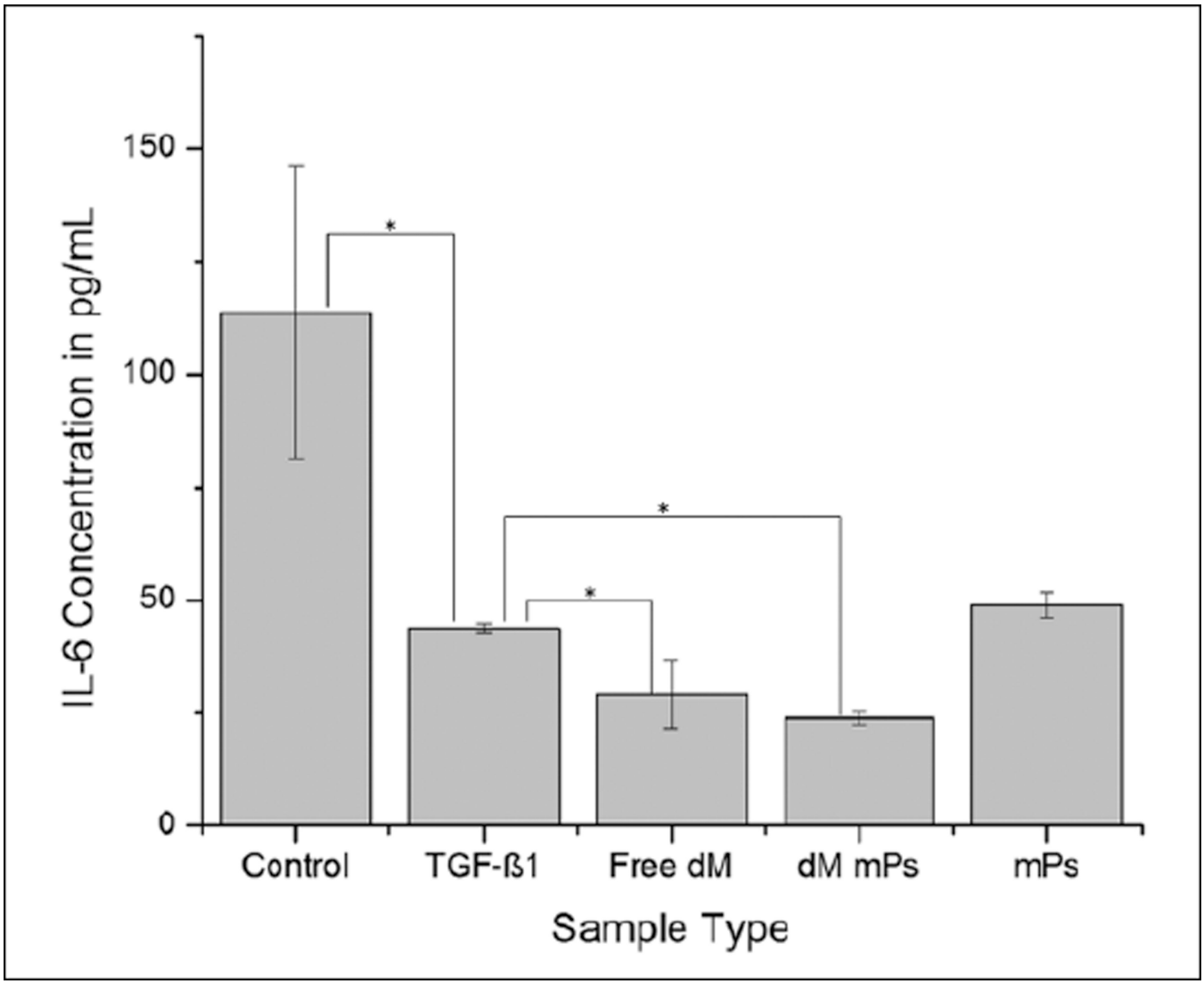

Figure 4.

IL-6 synthesis is significantly decreased by TGF-β1 treatment compared to control. Dexamethasone further knocks down IL-6 synthesis compared to TGF-β1 treatment alone. The greatest decrease is observed for ester-terminated low molecular weight poly-lactic-co-glycolic acid (PLGA) microparticles loaded with dexamethasone (dM mPs). *$P < .05$.