**Nano-drug delivery platform for glucocorticoid use in skeletal muscle injury.**

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Nano-drug delivery platform for glucocorticoid use in skeletal muscle injury.

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Abstract

Glucocorticoids are utilized for its anti-inflammatory properties in the skeletal muscle and arthritis. However, the major drawback with use of glucocorticoids is that it leads to senescence and toxicity. Therefore, based on the idea that decreasing particle size allows for increased surface area and bio-availability of the drug, in the present study we hypothesized that nano-delivery of dexamethasone will offer increased efficacy and decreased toxicity. The dexamethasone loaded PLGA (poly lactic-co-glycolic acid) nanoparticles were prepared using nanoprecipitation method. The morphological characteristics of the nanoparticles were studied under scanning electron microscope. The particle size of nanoparticles was 217.5±19.99 nm with polydispersity index (PDI) of 0.14±0.07. The nanoparticles encapsulation efficiency was 34.57±1.99% with in vitro drug release profile exhibiting a sustained release pattern over 10 days. We identified improved skeletal muscle myoblast performance with improved closure of the wound along with increased cell viability at 10nM nano-Dexamethasone-PLGA, however dexamethasone solution (1µM) was injurious to cells since the migration efficiency was decreased. In addition, the use of NP-Dexamethasone decreased LPS induced LDH release compared with dexamethasone solution. Taken together, the present study clearly demonstrates that delivery of PLGA-dexamethasone nano-particles to the skeletal muscle cells is beneficial for treating inflammation and skeletal muscle function.

Keywords: Dexamethasone, PLGA, Nanoparticles, skeletal muscle
**Introduction**

Dexamethasone and other glucocorticoids are used for the treatment of inflammation and pain related to skeletal muscle injury, wound healing and arthritis (Bijlsma 2012; Klein 2015; Vyvey 2010). However, the major problem associated with use of short and long term therapeutic use of glucocorticoids is that it causes muscle weakness and loss of muscle mass. Therefore, although dexamethasone and other GC’s provide a therapeutic advantage the issue of drug localization and toxicity need further understanding, and above all needs practical, applied solutions that will limit the toxicity of GC’s, while allowing its use. The use of novel drug delivery systems and nanotechnology based entrapment of glucocorticoids may offer advantages to overcome the therapeutic efficacy and dose, and therefore limit the toxic effects of GC’s. Previous studies using PLGA based nano particle allowed for high efficiency entrapment of drugs and slow sustained release of drug at site of injury (Mdzinarishvili et al. 2013). Based on this, in the present work we hypothesized that nano drug delivery of PLGA-dexamethasone will enhance efficacy and decrease toxicity.

Poly(lactide-co-glycolic acid) (PLGA) is an FDA-approved synthetic polymer that has been studied due to its biocompatibility. On the nanoscale this polymer has been shown to have controlled drug release, low cytotoxicity and few side effects (Hirani et al. 2016). The major benefits of applying nanotechnology to muscle drug delivery are: (1) size of particle allows for more patient-friendly administration, (2) smaller particles are compatible to the muscle, (3) nanoparticles are able to be manipulated by altering the weight and hydrophilicity, resulting in a more sustained release, (4) particles around 200 nm are able to be localized in the muscle cells (5) nanoparticles have increased solubility and surface area. The nanoparticles accumulates in injured tissues via enhanced permeation and retention (EPR) mechanism where vascular permeability is enhanced (Acharya and Sahoo 2011) (Dvir et al. 2011). The PLGA is a
biodegradable and nontoxic (Geldenhuys et al. 2011; Lu et al. 2009). It undergoes hydrolysis of its ester linkages in the presence of water to produce the original monomers, lactic acid and glycolic acid, which are byproducts of various metabolic pathways in the body under normal physiological conditions (Athanasiou et al. 1996). Recently, 4-AN loaded hydrogen peroxide-responsive copolyoxalate HPOX nanoparticles have been successfully investigated for ischemia–reperfusion injury (Lee et al. 2013) and elastin like nanoparticles for delivery of BMPs (bone morphogenetic proteins) and demonstrated induction of ALP activity and osteogenic mineralization in C2C12 cells (Bessa et al. 2010).

In this present study, we prepared a novel drug delivery system using dexamethasone loaded PLGA nanoparticles and investigated its physicochemical properties, particle size, zeta potential, encapsulation efficiency, differential scanning calorimetry (DSC), in vitro drug release, cell viability, molecular activity along with gene responses for evaluating the signaling to compare dexamethasone along with nano-particle delivery effects.

**Materials and Methods**

**Materials**

PEGA–COOH (copolymer ratio 50:50, M.W. 19,000) was received from, Akros Organic, NJ, USA. Dexamethasone hydrate was purchased from Sigma- Aldrich Co, St. Louis, MO, USA. Coumarin-6, DAPI and Phosphate buffered saline (PBS was purchased from Sigma-Aldrich Co, St. Louis, MO, USA). DMEM (Dulbecco's modified eagle medium, fetal calf serum and penicillín–streptomycin were purchased from Hyclone, Logan, UT, USA. Acetone, methanol and chloroform were purchased from VWR International, Batavia, IL, USA. All other chemicals used in the study were of analytical grade and were used without any further purification.
Preparation of Dexamethasone Nanoparticles

The nanoparticles were prepared using PLGA polymer using our previously reported nanoprecipitation method (Jinwal et al. 2013). Briefly, 2.8 mg of Dexamethasone and 14 mg of PLGA was dissolved in 1 ml acetone. The resulting solution was added dropwise at room temperature using a 16G needle to 2 mL of deionized water previously adjusted to a pH of 9.0 using sodium hydroxide (stirring at 350 rpm) to form nanoparticles. The acetone was allowed to completely evaporate overnight and the nanoparticles were collected by centrifugation at 5,000 rpm at room temperature for 20 min. The supernatant was removed and the nanoparticles were re-suspended in 3 ml deionized water. The final pH of the nano-dexamethasone preparation was 5.0 ± 0.1.

Determination of Particle Size

The size and polydispersity index (PDI) of the dexamethasone nanoparticles were analyzed through dynamic light scattering (DLS) using the DynaPro Plate Reader (Wyatt Technology, CA). The effect of drug loading on the size of the nanoparticles was observed by comparing the size and PDI of the drug-loaded NPs to essentially identically prepared blank PLGA nanoparticles. The nanoparticle samples were diluted 1:200 in filtered deionized water to fit equipment parameters.

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) Studies

JOEL JSM-6490 LV (JOEL Industries, Tokyo, Japan) was utilized to visualize the physical integrity of the nanoparticles and observe its basic ultrastructure. The samples were diluted according to instrumental specifications and were loaded onto aluminum cylinder coated with
adhesive carbon polymer. Nanoparticles were viewed at 65,000x magnification. 4 kv acceleration voltage was used to visualize the nanoparticles in 3 ml deionized water. The Dexamethasone loaded nanoparticles were also characterized for TEM in order to study the ultrastructure of the nanoparticles.

**Determination of Entrapment Efficiency**

A 0.70mL sample of dexamethasone nanoparticles was centrifuged at 12,000rpm for 5 minutes at room temperature. The supernatant was removed and replaced with 0.70mL of methanol and the sample was stored at 4°C overnight. The supernatant was analyzed via UV spectroscopy (λ= 242nm). By comparing this value to the absorbance of standard dilutions of dexamethasone in methanol (r²=0.9930), the drug content could be determined. The entrapment efficiency was determined using the following equation:

\[
\% \text{ Entrapment efficiency} = \frac{\text{Actual Drug Concentration}}{\text{Theoretical Drug Concentration}} \times 100\%
\]

**In vitro Drug Release**

The drug release profile of dexamethasone from the nanoparticles was investigated using our previously described method (Grover [In Press. Accepted 28th May, 2014]). A 0.5mL sample of dexamethasone nanoparticles were added to dialysis cassettes (MWCO 10,000 kDa) and placed in 100 mL of release medium comprised of PBS (pH 7.4) stirring at 100 rpm and 37°C. 1 mL aliquots were removed at predetermined intervals over 10 days and analyzed for dexamethasone content by UV spectroscopy (Cole Parmer, Vernon Hills, IL) at wavelength of 242nm. By
comparing this absorbance to standard dilutions of aqueous dexamethasone ($r^2=0.9914$) the cumulative percent of drug released at each time point could be determined.

**DSC Study**

Thermal analysis of pure dexamethasone and PLGA powders separately and in combination, as well as dexamethasone-loaded nanoparticles was performed using TA Instruments DSC Q 20 (TA Instruments, New Castle, DE USA). 1.8 mg of pure dexamethasone powder, 7.0 mg of pure PLGA, 6.5mg dexamethasone/PLGA combination, and 8.0 mg of dexamethasone -loaded nanoparticles were individually placed directly into a Tzero hermetic aluminum pan and pressed until thematically sealed. The samples were then separately heated from 30-300°C at a rate of 10°C min⁻¹ and cooled back from 300°C-30°C at a rate of 5°C min⁻¹ under a nitrogen flow of 50 mL/min.

**Cell Culture and Steroid Concentrations**

C2C12 and H9C2 cells were purchased from ATCC (Catalog #CRL-1772, CRL-1446). Cell culture plates and flasks were purchased from Corning Inc. (Corning, NY). 1% penicillin and streptomycin was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). PBS solution was purchased from Cellgro (Corning Inc., NY). C2C12 and H9C2 cells were cultured in 5% CO₂ incubator using standard DMEM medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin and streptomycin antibiotics. Dexamethasone (Dexa) was purchased from Sigma (dexamethasone-water soluble) with working concentrations of 1µM.

**Scratch Assay in C2C12 Cells**
C2C12 cells were seeded on a 12-well plate and allowed to grow to 80-90% confluence. Scratch or wound injury was induced by streaking the plate (2 streaks per well) between two previously marked measured lines using a 10 µL sterilized pipette tip in each well. The media was then removed and replaced with 1 mL media supplemented with either dexamethasone (1µM), nano-dexamethasone (10nM, 100nM) or control. Images were acquired immediately following the injury using an Evos core digital microscope and then 16 h post treatment. Closure assessment was quantified using ImageJ software, the 0 h area was measured and used as baseline and 16 h area measured and divided by the baseline.

**Intracellular Distribution of 6-coumarin Loaded NPs in H9C2 Cells**

H9C2 cells were seeded with 500µl DMEM containing 10% fetal bovine serum with penicillin (100 units/ml), streptomycin (100 mg/ml)) at 10,000 cells per well in an eight-well poly-D-lysine chamber slide. Cells were then incubated in humidified incubators with 5% CO₂ at 37 ºC for 24 h which resulted in 70-90% confluent cells. The medium was aspirated and the cells were washed with 1×PBS (phosphate buffer saline) three times. Then 500 µl of DMEM media containing 50 µg of 6-coumarin loaded nanoparticles (concentration of NPs: 15 mg/ml) was added to separate wells. The cultures were then incubated 5% CO₂ at 37 ºC for 2 h. The media was aspirated after 2 h and cells were washed three times with 1×PBS. The slide was then visualized by fluorescent microscopy with 20× magnification.

**MTT Assay in C2C12 Myotubules**

Briefly cells were seeded in a 96-well plate for 24 hours to achieve a stable confluency prior to treatment. Cells were exposed to dexamethasone (1µM) and nano-dexamethasone (10nM) for 24 hr after which the MTT assay was performed. Media was aspirated, and 100ul of MTT reagent
solution was added to each well, cells were incubated for 4 hrs after which reagent was aspirated and DMSO added to each well. Plate shaking occurred for 10 mins before reading by Synergy H4 plated reader (Biotek Industries, Inc., Winooski, VT) at absorbance of 570 nm.

**LPS Injury in C2C12 Myotubules and LDH Activity**

C2C12 cells were seeded into 12 well plates and differentiated utilizing standard DMEM media supplemented with 4% horse serum and 1% penicillin and streptomycin for 48 hours. Media was then removed and replaced with DMEM media supplemented with 4% horse serum and 1% penicillin and streptomycin and lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma) at 100ng/ml for 48 hours. Serum samples were collected after 48 hours of exposure and total lactate dehydrogenase activity was measured (LDH assay kit, Sigma).

**Quantitative Real-time PCR**

Total RNA was isolated from C2C12 myotubules using the Qiagen RNeasy isolation kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Complementary DNA from total RNA was synthesized, and quantitative real-time-PCR (qRT-PCR) analysis was performed. The cDNA synthesis and qRT-PCR procedures were performed as described previously. Cell cycle and inflammation genes such as FOXO1, P27, and Caspase-9 were utilized for our studies as genes of interest. The expression of mouse 18S (18S ribosomal RNA) was used as an internal control (Tur et al. 2017; Tur et al. 2016).

**Statistical analysis**

Statistical analyses were performed using the Student t-test and values of P ≤ 0.05 were considered to be statistically significant. Mean represents n=3-6 in each experimental group.
Results

Nanoparticle Size

The dexamethasone nanoparticles were 217.5 ± 19.99 nm in diameter with a polydispersity index (PDI) of 0.1438 ± 0.07. Similarly prepared blank nanoparticles demonstrated a diameter of 164.04 ± 13.24 nm and a PDI of 0.151 ± 0.0453 (figure 1). Poly dispersity index is another factor that represents the dispersion homogeneity. The range for the poly dispersity index is from 0 to 1. Values close to 0 indicates the homogenous dispersion and those greater than 0.5 indicate high heterogeneity. The PDI for the dexamethasone NP was ~0.14 which indicates homogenous dispersion. The zeta potential is an index of the NP’s stability and the higher zeta potential value represents larger charge on the particle surface and stronger repulsive interaction between the dispersed nanoparticles allowing higher stability and uniformity. The zeta potential of the dexamethasone nanoparticles and blank nanoparticle were -26.47 ± 0.551mV and -30.2 ± 1.65mV, respectively (figure 1). A high potential value of dexamethasone NPs above ±25mV ensures a high energy barrier that stabilizes the nanosuspension due to high energy barrier between the nanoparticles (Li et al. 2017b).

Entrapment Efficiency

The entrapment efficiency of the nanoparticles was determined via UV spectroscopy at 242nm. It was determined that 34.57± 1.99% of DEX used in preparation of the nanoparticles was encapsulated. The entrapment efficiency depended mainly on dexamethasone’s hydrophobic interactions with the hydrophobic core of the PLGA nanoparticles.

In Vitro Drug Release
The drug release from the nanoparticles depends on drug diffusion, PLGA surface and bulk erosion, or swelling (Mu and Feng, 2003) which is attributed to the slow degradation of the PLGA polymer. It was evident from the dexamethasone release curve that dexamethasone’s release from the PLGA nanoparticles was a biphasic release (Figure 1). The drug release involved an initial rapid release phase followed by a relatively slow release of the lag phase. The initial burst was due to the immediate dissolution and release of dexamethasone located near the surface and on the surface of the nanoparticles as suggested by others (Magenheim et al., 1993). It was found that around 13% of dexamethasone was released from the nanoparticles within 24 h. The nanoparticles showed sustained drug release over 10 days and approximately 100% of the drug was released by the aqueous solution within the initial 10 days of the study.

**DSC Study**

The DSC thermogram of pure dexamethasone powder indicated a melting point of approximately 280-290°C due to its observed endothermic peak. The PLGA powder showed a melting point of nearly 270°C. The physical mixture showed two peaks corresponding to dexamethasone (280-290°C) and PLGA (270°C). However, the DSC thermogram of dexamethasone loaded nanoparticles displayed a single endothermic peak at 43.17°C, thus indicating a change in the physicochemical properties of dexamethasone at the nano-level and indicated the absence of the drug crystalline state in the delivery system development (Figure 2).

**Closure Significant Hindered by Dexamethasone**

C2C12 cells which reached 80-90% growth confluence were “scratched” as detailed in methods. Cells were then exposed to dexamethasone or nano-dexamethasone solution for 16 hrs. Figure 3A demonstrates that control groups showed an average closure rate of 60% while exposure to 1µM dexamethasone significantly reduced the closure rate to 45% (Figure 3B). Therefore, a
significantly smaller area was closed in the presence of dexamethasone. Similar conditions were utilized for nano-dexamethasone resulted in a significant increase in closure rate compared with control group and 10 nM nano-dexamethasone with no decrease noted in 100 nM nano-dexamethasone (Figure 3C). The 10nM nano-dexamethasone may provide the beneficial effects of a glucocorticoid steroid while reducing the deleterious effect on cell growth and proliferation.

**Intracellular Uptake**

The intracellular uptake study of coumarin 6 loaded nanoparticles showed the green florescence of coumarin-6 from the cytoplasm and blue florescence from the nucleus due to DAPI labeling (Figure 4A-C). The uptake of nanoparticles by the cells is suggested to have occurred via endocytosis transport. The higher fluorescence intensity in cells treated with coumarin-6 loaded NP was due to the slow release of coumarin-6 from the encapsulate NPs while untreated cells did not show any auto florescence (Carroll et al. 2010).

**Scanning Electron Microscopy (SEM) Imaging**

Surface analysis of nanoparticles showed that physical integrity was maintained in the samples. Furthermore, the size, and size distribution of nanoparticles visualized through SEM corroborated with data obtained by DLS. (Figure 4D). The TEM micrographs also reflect nearly spherical in nature, more or less uniform fine particles (Figure 4D). The SEM study showed that the nanoparticles were spherical with smooth surfaces and solid dense with no aggregation which may be due to the high zeta potential on the surface of the nanoparticles, which helps to prevent the agglomeration process.

**MTT Analysis**
The MTT analysis performed with dexamethasone and nano-dexamethasone indicates that there are no significant effects of these compounds on the metabolic activity of the cells (Figure 5A). Cell survival was not significantly altered in C2C12 cells differentiated into myotubes through induction with horse serum (4%), suggesting no significant detrimental or toxic effects from these compounds (Figure 5A).

**LPS Exposure Increases Lactate Dehydrogenase (LDH) Release**

C2C12 cells differentiated into myotubes for 48-96 hours after induction with horse serum (4%) were exposed to LPS (100ng) for 48 hours. Simultaneously cells were also exposed to dexamethasone (1µM) or nano-dexamethasone (170nM) for 48 hours. LPS treatment significantly increased LDH release from cells after 48 hours of exposure; however the addition of dexamethasone (1µM) as well as nano-dexamethasone (170nM) significantly reduced the LDH release (Figure 5B). Interestingly dexamethasone (1µM) significantly attenuated the LDH release compared with no LPS group, while nano-dexamethasone treatment decreased the LDS levels similar to control. These data clearly points to the beneficial effects of nano-dexamethasone based the reduction of LDH levels primarily caused inflammation.

**Quantitative Real-Time PCR**

C2C12 myotubes exposed to LPS as well as dexamethasone and nano-dexamethasone were collected and RNA isolation performed for qRT-PCR analysis. LPS treatment showed a significant increase in FOXO1, P27 and Caspase 9 when compared with control (Figure 6). In contrast, LPS exposure along with either dexamethasone or nano-dexamethasone resulted in significant reductions in FOXO1, P27 and Caspase9 genes upregulated by LPS.
Discussion

The present study utilized nanoprecipitation to formulate dexamethasone loaded nanoparticles with the synthetic polymer, poly lactic-co-glycolic acid (PLGA). The encapsulation efficiency of these nanoparticles was found to be 34.57± 1.99%. This may be further optimized by altering the polymer utilized in formulation as well as the specific formulation process. SEM imaging showed the dexamethasone loaded nanoparticles had an average diameter of 147.37 nm with no observable aggregation and a uniform, spherical morphology. Drug localization in the inner ocular structures is ideal when the drug carrier is between 100-200 nm (Hirani et al. 2016). Therefore, dexamethasone loaded nanoparticles were within the ideal size range for nanoparticle delivery for skeletal muscle injury application. Further confirming uniformity of size distribution, the PDI was found to be consistently below 1 (0.1438 ± 0.07), thus indicating a reasonably homogenous dispersion. As previously stated, the DSC thermogram of pure dexamethasone powder showed a peak at approximately 280-290°C, indicating its melting point. The melting point of pure dexamethasone powder was consistent with literature 262-264°C. Dexamethasone melting peak disappeared in the DSC thermogram of drug-loaded in PLGA nanoparticles, indicating the absence of the drug crystalline state in the delivery system development. Nanoparticles with a zeta potential of ± 25 mV are considered to be highly stable (Jo et al. 2015). The surface charge of the dexamethasone loaded nanoparticles was determined to be -26.47 ± 0.551 mV, thus substantiating the positive interaction between the nanoparticles with muscle cells. Comparing the cumulative drug release (%) of dexamethasone loaded nanoparticles against free dexamethasone solution showed that the nano-based delivery system provided a significant increase in sustainability.
We identified that nano dexamethasone drug delivery is beneficial to the skeletal muscle as an anti-inflammatory agent at a much lower concentration. In addition, the major issue with the use of corticosteroid for skeletal muscle conditions is limited due to senescence which can likely be overcome by use of much lower concentrations with nano drug delivery for sustained and effective drug release. The use of nano-technology based dexamethasone particles allow for nano molar level of drugs to be utilized and therefore under injury conditions cells grow and retain their ability to cover the injured part in a highly efficient manner. We identified that nano molar use of dexamethasone as nanoparticle drug delivery provides the ability for C2C12 cells to migrate and overcome cell senescence. Dexamethasone and other glucocorticoids impair cell growth and proliferation (Zhao et al. 2008) whereas we observe that by decreasing the concentration and improving delivery of dexamethasone preserved growth and enhanced cell repair machinery. In addition using MTT assay we evaluated cytotoxicity in C2C12 cells and observed overt toxicity with nano-dexamethasone delivery to cells. In an attempt to gain some insights into the molecular machinery underlying the effects of dexamethasone in muscle cells, we measured key molecular markers by RT-PCR. Cell senescence, apoptosis and proliferation status were assessed via mRNA measurement of FOXO1, caspase 9, and p27.

The loss of muscle mass and myofibrilar proteins known as muscle atrophy can result from multiple pathologies including infection and inflammation. LPS-induced muscle atrophy is a reliable model for identifying the muscle injury in the in vitro conditions, FOXO1 expression (Liu et al. 2013) in gastrocnemius muscle has been demonstrated to increase under inflammatory conditions, therefore we utilized C2C12 cells and under in vitro conditions to exposed the cells to LPS induced damage resulting in changes in FOXO1 mRNA expression. Based on the cell responses we identified that while LPS caused a significant increase in FOXO1 expression,
treatment with dexamethasone and nano-dexamethasone significantly reduced FOXO1 expression. Previous reports show that aspartate inhibits LPS induced MuRF1 via Akt along with inhibition of FOXO1 signaling. Results from the present study are consistent with this and identify that while FOXO1 is upregulated after LPS treatment however dexamethasone decreases the FOXO1 expression identifying that dexamethasone effects may regulate inflammatory stress, cellular differentiation and development (Lu and Huang 2011). To identify the gene expression of caspase-9 a key marker for apoptosis in cells (Li et al. 2017a), LPS induced expression was evaluated in C2C12 cells. LPS induced increase in caspase-9 expression which was significantly decreased with dexamethasone treatment at 1µM and with lower concentration nano dexamethasone treatment (10nM). The cell cycle marker P27 was evaluated under LPS induced conditions, since P27 which is cyclin dependent kinase (CDK) inhibitor (CKI) has been previously reported to regulate CDK at the cell cycle G1/S phases (Moller 2000). LPS is an inflammatory inducer and leads to cell differentiation, and we observed that dexamethasone treatment leads to decrease in LPS induced inflammation in C2C12 cells and the nano delivery of dexamethasone allowed for a decrease in concentration required to attenuate the expression of P27 in the C2C12 cells. Overall, at the molecular level we demonstrate that cell death, differentiation, apoptosis and senescence is attenuated with dexamethasone treatment and dexamethasone offers beneficial protective effects against LPS induced inflammation in skeletal muscle myoblast cells. The anti-inflammatory role of dexamethasone was evaluated using the LPS induced LDH release in undifferentiated C2C12 cells. While dexamethasone inhibited the LPS induced LDH release in C2C12 cells, the advanced drug delivery of PLGA nano-particles of Dexamethasone lowered the concentration required for dexamethasone used for similar effect of
LDH decrease in C2C12 cells. This mode of drug delivery readily offers enhanced therapeutic efficacy with lower side effects that limits the use of glucocorticoids in skeletal muscle injuries.

**Conclusions**

A novel biodegradable dexamethasone loaded PLGA nanoparticles system was synthesized for effective delivery of the dexamethasone into the skeletal muscle. Particle sizes were in the range of 50-200 nm, reproducible and suitable for the skeletal muscle. The nanoparticles showed a sustained drug release behavior. Cellular uptake study in C2C12 cells showed excellent uptake of the nanoparticles. The SEM and TEM studies showed the spherical nature and good uniformity of the particles. The proposed dexamethasone nanoparticles could be used for the effective treatment of skeletal muscle inflammation and other musculoskeletal diseases.

**Acknowledgements**

Authors acknowledge the Florida High Tech Corridor funding support.
Figure Legends:

Figure 1. Nano particle drug delivery and characterization of PLGA-dexamethasone:  A. Particle size measurement,  B. Zeta potential measurement in blank nano particle or dexamethasone nanoparticle inset is representative of zeta potential measurement demonstrates the zeta potential distribution in the nanoparticle preparation,  C. Polydispersity index (PDI) measured using blank nanoparticle lacking the active ingredient, or nanoparticle with dexamethasone, inset provides original measurement for PDI depicting characteristics for size (radius in nanometers, % intensity).  D. In vitro drug release data of Dexamethasone Nanoparticles. The nanoparticles exhibited sustained release of drug for 10 days.

Figure 2. Differential Scanning Calorimeter (DSC) thermograms of the pure dexamethasone (DEX), PLGA, DEX PLGA Physical Mixture, and DEX-loaded PLGA NPs. Free DEX exhibited endothermic melting peak approximately at 280-290°C, typical of a drug in the crystalline form. The PLGA powder showed a melting point of nearly 270°C. DEX melting peak disappeared in the DSC thermogram of drug-loaded in PLGA nanoparticles, indicating the absence of the drug crystalline state in the delivery system development.

Figure 3. Effect of glucocorticoids on skeletal muscle cells wound closure  A. Representative images of C2C12 (murine skeletal myoblasts) after wire injury and treatment with glucocorticoids (dexamethasone, Dexta) for 16 hrs. Black lines indicate the area measured. Wound infiltration was measured by image J.  B. The extent of wound closure 16 hrs after initial cell injury is expressed as a percentage of basal control (vehicle treated) vs. dexamethasone (1µM).  C. The extent of wound closure 16 hrs after injury is expressed as a percentage of basal (vehicle) vs nano-dexta 10 nm or nano-dexta 100nM. Data presented as mean ± SEM, *P<0.05.
Figure 4. PLGA-Nanoparticle uptake and dexamethasone drug physical characterization. A - C. Uptake of PLGA coumarin nano formulation Representative images of H9C2 (rat cardiomyoblasts) cells after 30 min exposure to coumarin 6 nanoparticles (right image) demonstrated uptake of the coumarin 6 within the cells. D. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) characterization of dexamethasone nanoparticles.

Figure 5. Effect of dexamethasone and nanoparticle dexamethasone on cell viability and LPS induced LDH release on skeletal muscle cells and. A. Differentiated C2C12 cells incubated with dexamethasone, DEX (1µM); and Nano-dexamethasone, Nano-DEX (10nM) for 24hrs were assayed for survival and viability. MTT assay was measured and data are mean ± SEM, *P<0.05. B. C2C12 cells incubated in 2% horse serum differentiated into myotubes and were subsequently exposed to LPS (100ng/ml) for 48 hrs with control (media), dexamethasone, DEX (1µM); Nano-dexamethasone (170nM was calculated based on entrapment efficiency). Post exposure cell serum was collected and LDH (lactate dehydrogenase) release/activity of C2C12 cells were measured. LDH activity in serum was measured and data are mean ± SEM, *P<0.05.

Figure 6. Gene expression changes caused by LPS injury and dexamethasone treatment. C2C12 cells exposed with LPS were treated with dexamethasone or nano-dexamethasone to identify qRT-PCR based gene expression changes. A. FOXO1, B. p27, and C. Caspase 9. Comparison between LPS (-) compared with LPS treated C2C12 cells and rescue by dexamethasone (Dexa) and nano-dexamethasone (Nano-Dexa) treatment. Data presented as mean ± SEM, *P<0.05.
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Figure 1

A. Bar chart showing particle size (nm) for Blank and Dex NP.

B. Bar chart showing zeta potential (mV) for Blank and Dex NP.

C. Bar chart showing polydispersity index for Blank and Dex NP.

D. Graph showing drug released over time (hours) for DEX Solution and DEX NPs.
Figure 3

A. 0 Hr 16 Hr

Control

Dexa (1µM)

B. 

C. 

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**Figure 4**

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H9C2

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Dex NP

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Figure 5

A. C2C12-Myotubes

MITT Assay % Cell Survival

Control | Dixa (1µM) | Nano-Dexa (10nM)

B. LDH (milliunit/mL)

LPS | + | + | +

Dexa (1µM) | | + | |

Nano-Dexa (170nM#) | | | +

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Figure 6

**FOXO1**

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<th>Condition</th>
<th>Average Fold</th>
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<td>Nano-Dexa</td>
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**p27**

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**Caspase 9**

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