Characterization of synthesized NANO-encapsulated drug for bone loss on hind limb suspension rat model by NMR and micro-CT

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Abstract: A formulation of nano-encapsulated enantiomer of (+) promethazine with desired release rate has been synthesized for establish a localized drug delivery system. It was tested on a hind limb suspension (HLS) disuse rat model, and by using a non-destructive Nuclear Magnetic Resonance (NMR) relaxation technique, and micro computed tomography (Micro-CT) analysis technique to qualitatively evaluate the effectiveness of the new bone formations as well as to compare the current commercial anti-bone loss drug Alendronate. Our studies suggest that nano-encapsulated (+) promethazine in controlled release formulations conjugating bone-targeting functional groups are effective in promoting bone growth in a disuse rat model.

Keywords: Nano-Encapsulation, Bone, NMR, Micro-CT

1. Introduction

Bone loss, osteoporosis, is recognized as a significant major public health problems as well as in the space programs worldwide. Osteopenia is a disease characterized by long term loss of bone tissue, particularly in the weight-supporting skeleton [1-2]. Results of the joint Russian/US studies on the effect of microgravity on bone tissue from 4.5- to 14.5-month long missions have demonstrated that bone mineral density (BMD, g/cm²) and mineral content (BMC, g) are diminished in all areas of the astronaut skeleton [3]. While osteopenia can affect the whole body, complications often occur predominantly at specific sites of the skeleton with great load bearing demands. The greatest BMD losses have been observed in the skeleton of the lower body, i.e., in pelvic bones (-11.99±1.22%) and in the femoral neck (-8.17±1.24%), while there was no apparent decay found in the skull region. On average, the magnitude and rate of the loss is staggering; astronauts lose bone mineral in the lower appendicular skeleton at a rate approaching 2% per month [4-5]. Similar results were found in the bed rest study. In a -6 degrees head-down tilt 7-day bed rest model for microgravity, it was observed that there was a decreased bone formation rate in the iliac crest [6]. To effectively countermeasure the bone loss, we need a better therapeutic system that can deliver the treatment in a need-base and non-invasively. An adequate understanding of the underlying mechanism and treatment strategy of such skeletal complications are extremely needed.

Most bone tissue turnover occurs at bone surfaces, such as at the interface to the marrow or in Haversian systems. Bone surfaces are normally covered by lining cells. In response to resorption stimuli these lining cells retract and expose the bone surface to attachment by osteoclasts and subsequent bone turnover. Therefore, targeting to the calcified matrix is most likely to occur at sites of active resorption. Bisphosphonates exhibit exceptionally high affinity to bone mineral hydroxyapatite; using an inactive bisphosphonate moiety deliver NANO-encapsulated (+) promethazine provide a promising method to treat a variety of bone diseases [7-12]. Promethazine, an H1 receptor blocker phenothiazine, was found to inhibit age-related bone loss in animal studies [13]; and the (+) enantiomer of promethazine was found to have a threefold higher efficacy
for osteoclast inhibition than both the racemate and the (-)
enantiomer [14].

The objective of this research study is to establish a
unique localized drug delivery system for bone loss in the
critical region by developing a NANO-encapsulated
medicine protocol to accelerate local therapeutic effects,
and by using a non-invasive NMR relaxation technique,
and micro-CT analysis technique to evaluate the
effectiveness of the formation as well as to compare the
current commercial anti-bone loss drug Alendronate. In this
program we have developed effective, less toxic
NANO-encapsulated drug, and control the release rate of
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2. Experimental Section

2.1. Animal Model and Sample Population

A disuse animal model is developed through generated
reduced or zero lower limb weight-bearing disuse hind limb
suspension (HLS) rat model with age of 5 months [24]. Rat
femurs obtained from the Department of Biomedical
Engineering, SUNY Stony Brook, New York through a
collaborative relationship between SUNY and Southwest
Research Institute (SwRI). Animal experiment was guided
by the IACUC. HLS preparations were initially performed
on the two tests. In each test the adaptive responses were
evaluated following four weeks period on 6 month female
rats. The test was applied on total of 6 groups (N=5 per
group) in female rats: 1) disuse sham, 2) disuse plus drug of
035-P18, 3) disuse plus drug of 035-P21, 4) disuse plus
Alendronate, 5) age match normal control, and 6) baseline
control (animals’ bone harvested at the beginning of the
experimental period) (Table 2). A preliminary study (test 1)
was performed prior to this reported study, in which the
dosage of the drug was at 0.33mg/kg body weight. The
results showed less effective and insignificant. Based on this
preliminary test, the drug dosage of 0.66 mg/kg body weight
every two days, IP injection) was selected in the second test
reported in this study. All the samples (right legs) were
cleaned of soft tissues, and wrapped in calcium gauze and
stored in separate containers filled with calcium buffered
saline (CBS) and frozen at approximately -20°C until
testing.

2.2. NMR Determination

It is known that Nuclear Magnetic Resonance (NMR)
proton spin-spin (T2) or spin-lattice (T1) relaxation time
measurements and analytical processing techniques have
been used to determine microstructural characteristics of
various types of fluid filled porous materials with
characteristic pore sizes ranging from sub-micron to
sub-millimeter [15-18]. Currently this method has been
developed and applied to quantify on human cortical bone
[19-23]. The observed proton NMR relaxation signals are a
convolution of the relaxation of fluid in the pores
throughout the observed system with the longer relaxation
time corresponding to larger pore sizes [27].

2.2.1. NMR Measurement

A home-built 0.5 to 40 MHz broadband NMR system
with an electromagnet of 19 inch diameter with a 4 inch
gap was set up for a proton frequency of 27 MHz for these
measurements. A laboratory-built 1.0 inch diameter rf coil
was used in the experiment. 1H spin-spin (T2) relaxation
profiles were obtained by using NMR CPMG [25-26]900
- 1800 – τ (echo)|n – TR} spin echo method with a 6.5
µs wide 90o pulse, τ of 500 µs, and TR (sequences
repetition rate) of 15 s. Each T2 profile, one thousand
echoes (one scan with n = 1000) were acquired and forty
scans were used. Thus, one scan has repeated 1000 echoes
in the window. The data was measured from fresh frozen
rat bone after complete thawing in the room temperature
(21 ± 1°C).

2.2.2. The Relationship between NMR Data and Effective
Pore Sizes

Based on the low field NMR principle the diffusion
effect may be negligible. Here, we accept the Brownstein
and Tarr assumption [27] that the relaxation rate 1/T2 is
proportional to the surface-to-volume (S/V) ratio of the
pore

\[
1/T2 = \rho \ (S/V)_{pore}
\]

where ρ is the surface relaxivity, which is a measure of the
effects of the pore surface enhancing the relaxation rate.
Equation (1) indicates that the NMR relaxation time is
proportional to pore size.

For a porous bone, the observed NMR magnetization
will depend upon the T2 of broad distributions of water in
all pores. This implies that NMR transverse relaxation (T2)
data can be expressed as a sum of exponential functions:

\[
M(t_i) = \sum_{j=1}^{m} f(T_{2,j}) \exp(-t_i/T_{2,j})
\]

where f (T_{2,j}) is proportional to the number of spins which
relax with a time constant T_{2,j}. M(t_i) is the NMR
magnetization decay from fluid saturated cortical bone.
Equation (2) can be inverted into a T2 relaxation time
distribution. Thus, instead of estimating a single relaxation
time from a magnetization decay, it is necessary to estimate
an inversion T2 spectrum or distribution of relaxation time
f(T_{2,j}), and an inversion relaxation technique was applied
[15-16, 19, 20, 23]. Since T2 depends linearly upon pore
size, the T2 distribution corresponds to pore-size
distribution with the longer relaxation times having the
larger pores. In addition, the median T2 from the T2
relaxation distribution can provide the overall effective
bone pore size, i.e., the quality of whole bone. These results
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are also compared with microCT data.

2.2.3. Median T2 Relaxation Time

The median T2 relaxation calculation is based on T2 relaxation distribution data. In T2 relaxation distribution spectra, the water intensity (amplitude in y axis) is plotted against T2 relaxation time (x-axis) which corresponds to different pore sizes and the cumulative water intensity amplitudes, and is normalized to 1. Therefore, the middle point 0.5 on y axis corresponds to the median relaxation time on x-axis (Figure 3). This median relaxation time method can provide the relaxation mechanism for whole bone without considering the bone size difference, i.e. different bone volume for different bone.

2.3. MicroCT Analysis

A high resolution microCT scanner (microCT-40, SCANCO Medical AG, Bassersdorf, Switzerland) was used, and the samples were scanned with a spatial resolution of 15 µm. In basic bone research bone volume density (BV/TV), trabecular spacing (Tb.Sp.), and trabecular number (Tb.N) are key measurements characterizing the three-dimensional (3D) structure of bone by microCT technology. BV/TV indicates the fraction of a given volume of interest (VOI, i.e. the Total Volume TV) that is occupied by mineralized bone (Bone Volume). BV/TV is usually reported as a % value and it can be used to evaluate relative changes in bone volume density following a given treatment. One way to determine the efficacy of the drug is to compare BV/TV in defined VOIs in control (or negative control group) vs treatment group. You would expect that if the drug works, the treatment group shows higher BV/TV values than the HLS group. Trabecular separation (Tb.Sp.mm) is essentially the thickness of the spaces as defined by binarisation within the VOI. It can also be calculated wither from 2D images with model assumptions or directly in 3D. Trabecular number (Tb.N. 1/mm) implies the number of traversals across a trabecular or solid structure made per unit length on a linear path through a trabecular bone region. The concept for measuring the average trabecular the average separation and average number can be applied to any structure and used in many different fields.

3. Results and Discussion

3.1. Synthesis of PLGA Nanoparticles with Encapsulated (+) Promethazine

From our study the nanoparticles of (+)promethazine in block copolymers of poly (ethylene glycol)-b-poly(lactic acid glycolic acid) (PEG-PLGA) PLGA were successfully prepared with about 2% payload and an encapsulation efficiency of 40% by a double emulsion method [28]. The nanoparticle size distributions are shown in Figure 1. The positively charged nanoparticle samples demonstrated controlled release of (+)promethazine for a day (Figure 2). The lyophilized nanoparticles can be re-suspended in pH7.4 PBS.

Three nanoparticle samples were prepared for in vivo testing. Two encapsulated drugs with different concentrations of (+)PMZ, and one with pure Alendronate sodium were successfully synthesized. The details of the drug samples are shown in Table 1 below.

After the data analysis from initial test (drug dose 0.33 mg per rat body weight) it was selected the drug dose (0.66 mg/kg) for the following test protocol in Table 2 on second test.

3.2. NMR Results

It is found that the median T2 relaxation time as measured by NMR is one of the best parameters for whole bone (includes cortical, trabecular, and mallow) porosity evaluation, it can be used to effectively determine bone quality changes under various testing conditions for the animals (e.g. HLS, HLS + drug, and normal only) . The median T2 relaxation calculation is based on T2 relaxation distribution data. The middle point 0.5 on y axis corresponds to the median relaxation time on x-axis (Figure 3). This median relaxation time method can provide the whole relaxation mechanism without considering the bone size difference, i.e. different bone volume for different bone. It is also a sensitive method to analyze all pore size changes in an entire bone.

In figure 3, the longer relaxation time corresponds to larger pore sizes and the higher intensity corresponds to larger pore volume. Therefore, it is assumed that the first peak (from left to right) mainly is contributed from the cortical bone region, the second peak is mainly contributed from cortical bone and bone marrow, and the third peak is mainly contributed from the trabecular region.

A summary of the median T2 relaxation times obtained from the in vivo animal studies (at normal, HLS only, HLS+drug alendronate, HLS+drug 035-P18, and HLS+drug 035-p21 are listed in Table 3. It is suggested that on average the drug 035-p18 improves significantly over the disuse HLS with better than drug 035-p21, and slightly better than the currently in commercial used alendronate.
Figure 2. In vitro test for controlled release of nanoparticles of (+)promethazine.

Table 1. Sample Detail

| Sample No. | Weight (mg) | Composition       |
|------------|-------------|-------------------|
| 10-0203-035-p18 | 481         | 14% (+)PMZ/86% PLGA-10%PEG |
| 10-0203-035-p21 | 632         | 40% (+)PMZ/60% PLGA-10%PEG |
| Alendronate (ALN) | 500           | 100% Alendronate sodium trihydrate |

Table 2. Test Protocol

| Group | Group Description | Animals |
|-------|-------------------|---------|
| 1     | Age-Matched Control Only | 5       |
| 2     | Hind-limb Suspension Only | 5       |
| 3     | Hind-limb Suspension + DRUG (035-p18) | 5       |
| 4     | Hind-limb Suspension + DRUG (035-p21) | 5       |
| 5     | Hind-limb Suspension + Alendronate (ALN) | 5       |
| 6     | Baseline Control (bone samples harvested at day 0) | 5       |

Figure 3. NMR T2 relaxation time distribution and median T2 relaxation for sample A10 (normal).

Table 3. Median Relaxation Times listed for Different Group Samples

| Sample # (Normal) | Median relaxation (ms) | Sample # (HLS) | Median relaxation (ms) | Sample # (HLS+drug 035-P18) | Median relaxation (ms) |
|-------------------|------------------------|----------------|------------------------|-----------------------------|------------------------|
| A6                | 56.80                  | A11            | 56.21                  | A20                         | 56.26                  |
| A7                | 57.68                  | A12            | 61.43                  | A21                         | 51.70                  |
| A8                | 48.64                  | A13            | 59.82                  | A22                         | 54.97                  |
| A9                | 53.36                  | A14            | 61.88                  | A23                         | 54.56                  |
| A10               | 51.20                  | A15            | 58.58                  | A24                         | 58.40                  |
| Average           | 53.54±3.78            |                | 59.58±2.30             | 55.18±2.45                  |                        |

| Sample # (HLS+drug 035-P21) | Median relaxation (ms) | Sample # (HLS+drug 035-P18) | Median relaxation (ms) |
|-----------------------------|------------------------|-----------------------------|------------------------|
| A26                         | 54.94                  | A32                         | 54.44                  |
| A27                         | 55.20                  | A33                         | 58.03                  |
| A28                         | 49.44                  | A34                         | 60.62                  |
| A29                         | 51.84                  | A35                         | 59.93                  |
| A30                         | 54.62                  | A36                         | 56.42                  |
| Average                     | 53.21±2.50            |                            | 57.88±2.53             |
3.3. Micro-CT Results

Throughout the entire experimental period, the animals’ body weight was monitored. The body weights were not significantly different between groups at the beginning of the study, with an average of 320±47 g.

From the data below, the lack of weight-bearing activity for 4 weeks significantly reduced trabecular bone quality and quantity, in the distal metaphyseal region directly above the growth plate demonstrated by a 34.7% decreases in the average of BV/TV, a 18.7% decreases in the average of Tb.N, and a 28.2% increases in the average of Tb.Sp comparing between HLS and No HLS control groups. Summaries of microCT measurements are in the following Tables and corresponding Figures 4-6. Here, in Tables the columns 1 to 6 are counted from left to right corresponding to the relative Figures.

Figure 4. Bone volume fraction (BV/TV) changes among control, HLS and HLS plus different drugs.

Figure 5. Bone trabecular number (Tb.N, 1/mm) changes among control, HLS and HLS plus different drugs.

Figure 6. Bone trabecular separation (Tb.Sp, mm) changes among control, HLS and HLS plus different drugs.

### Table 4. Summary (BV/TV)

| Groups  | Count | Sum  | Average | Variance |
|---------|-------|------|---------|----------|
| Column 1 | 5     | 0.8552 | 0.2138 | 0.002748 |
| Column 2 | 5     | 1.3655 | 0.2731 | 0.003962 |
| Column 3 | 5     | 1.4260 | 0.1783 | 0.005160 |
| Column 4 | 5     | 1.0017 | 0.2504 | 0.008963 |
| Column 5 | 5     | 1.5320 | 0.3064 | 0.02034 |
| Column 6 | 5     | 1.1219 | 0.2244 | 0.007890 |

### Table 5. Summary (Tb.N)

| Groups  | Count | Sum  | Average | Variance |
|---------|-------|------|---------|----------|
| Column 1 | 5     | 15.5490 | 3.8873 | 0.3399 |
| Column 2 | 5     | 23.5018 | 4.7004 | 0.5415 |
| Column 3 | 5     | 30.5813 | 3.8227 | 1.0944 |
| Column 4 | 5     | 18.5307 | 4.6327 | 0.8616 |
| Column 5 | 5     | 21.9777 | 5.4944 | 0.0444 |
| Column 6 | 5     | 20.5668 | 4.1133 | 0.6592 |

### Table 6. Summary (Tb.Sp)

| Groups  | Count | Sum  | Average | Variance |
|---------|-------|------|---------|----------|
| Column 1 | 5     | 1.0643 | 0.2661 | 0.002490 |
| Column 2 | 5     | 1.0604 | 0.2121 | 0.001110 |
| Column 3 | 5     | 2.2596 | 0.2825 | 0.007691 |
| Column 4 | 5     | 0.8992 | 0.2248 | 0.002380 |
| Column 5 | 5     | 0.6891 | 0.1723 | 2.080E-05 |
| Column 6 | 5     | 1.2174 | 0.2435 | 0.002618 |

4. Conclusions

The results from our studies are positive. It is suggested that nano-encapsulated (+)promethazine in controlled release formulations conjugating bone-targeting functional groups can be effective in anti-bone loss in a HLS rat model. The improvement is quite significant, especially, for the drug sample of 035-p18. The test results show that after
the treatments on HLS group, the average bone porosity values were approachable or better than those of the control (normal) groups. It was demonstrated the (+)PMZ can be functional as anti-bone loss at microgravity condition and other similar conditions. During the experiment a preliminary test (test 1) was performed prior to this reported study, in which the dosage of the drug was at 0.33mg/kg body weight and the results showed the changes among HLS and HLS+drugs (HLS+alendronate, HLS+drug 035-p18, and HLS+035-p21) less effective and insignificant. Based on this preliminary test, the drug dosage of 0.66 mg/kg body weight was selected in the second test reported in this study. This dosage selection is according to Promethazine for human oral is 25–50 mg per time, and for per Kg mass is 25-50 mg/75kg = 0.33-0.66 mg/kg. Therefore, the certain amount of drug dosage is needed to insure anti-bone loss function of the drug.

The second HLS test showed a very positive result as we reported, i.e. the significant function of anti-bone loss. The results indicate that the formulated drug (035-p18) has the independent function similar or better than commercial market product-Alendronate that is used for bone loss treatment. It is clearly to show that (+)PMZ has the significant anti-bone loss function, it is also suggested applying both (+)PMZ and Alendronate may produce the further improvement for bone loss.

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