AD_____

Award Number: W81XWH-10-1-0853

TITLE: Anabolic Steroids as a Novel Therapeutic Strategy for the Prevention of Bone Loss after Spinal Cord Injury: Animal Model and Molecular Mechanism

PRINCIPAL INVESTIGATOR: Weiping Qin, MD.,Ph.D

CONTRACTING ORGANIZATION: Bronx Veterans Medical Research Foundation Inc. Bronx, NY 10468

REPORT DATE: October 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2012	Final	30 September 2010- 29 September 2012

4. TITLE AND SUBTITLE
Anabolic Steroids as a Novel Therapeutic Strategy for the Prevention of Bone Loss after Spinal Cord Injury: Animal Model and Molecular Mechanism

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0853

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Weiping Qin, MD.,Ph.D

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Bronx Veterans Medical Research Foundation Inc.
Bronx, NY 10468

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Background: Spinal cord injury (SCI) causes severe bone loss. At present, there is no practical treatment to delay or prevent bone loss in individuals with motor-complete SCI. Hypogonadism is common in men after SCI and may exacerbate bone loss. The anabolic steroid nandrolone reduces bone loss due to microgravity or nerve transection.
Objective: To determine whether nandrolone reduced bone loss after SCI and, if so, to explore the mechanisms of nandrolone action.
Methods: Male rats with complete transection of the spinal cord were administered nandrolone combined with a physiological replacement dose of testosterone, or vehicle, beginning on day 29 after SCI and continued for 28 days
Results: SCI reduced distal femoral and proximal tibial bone mineral density (BMD) by 25 and 16%, respectively, at 56 days. This bone loss was attenuated by nandrolone. In ex vivo osteoclasts cultures, SCI increased mRNA levels for tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor; nandrolone-normalized expression levels of these transcripts. In ex vivo osteoblast cultures, SCI increased receptor activator of NF-kB ligand (RANKL) mRNA levels but did not alter osteoprotegerin (OPG) mRNA expression; nandrolone-increased expression of OPG and OPG/RANKL ratio. SCI reduced mRNA levels of Wnt signaling-related genes Wnt3a, low-density lipoprotein receptor-related protein 5 (LRP5), Fzd5, Tcf7, and ectodermal-neural cortex 1 (ENC1) in osteoblasts, whereas nandrolone increased expression of each of these genes.
Conclusions: The results demonstrate that nandrolone reduces bone loss after SCI. A potential mechanism is suggested by our findings wherein nandrolone modulates genes for differentiation and activity of osteoclasts and osteoblasts, at least in part, through the activation of Wnt signaling.

15. SUBJECT TERMS- Spinal cord injuries, Nandrolone, Androgens, Hypogonadism, Bone loss, Wnt signaling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

During two years of funding, we have conducted a series of experiments with three major goals to address research tasks that we have proposed in DOD Award (10-1-0853); we also have successfully conducted one additional but very relevant study. 1) We have studied the anabolic effect of nandrolone in spinal cord injury (SCI) associated bone loss and a possible underlying mechanism of such effects; 2) We have evaluated two new models of SCI-related bone loss in male rats and established a novel model of SCI-related bone loss in male rats; 3) We have established in vitro cell culture systems and approaches feasible to study effects of nandrolone on the extent of differentiation of osteoblasts and osteoclasts and we performed some initial studies using these techniques. In ancillary studies using the animal model developed through the DOD-funded work and performed in collaboration with Amgen (a leading pharmaceutical company), we have conducted a pilot study to evaluate the efficacy of an anti-sclerostin antibody to prevent bone loss after acute SCI in rats. Excitingly, we have found that the Scl-Ab almost completely prevented SCI-induced bone loss acutely after injury.

Body

Task 1: Establish a novel model of SCI-related bone loss in male rats.

We evaluated bone loss and the effects of nandrolone on such loss using a novel hemisection of SCI in male rats based on a model developed by Ted Teng which had the advantage of rapid return of automatic emptying of the bladder. The surgery entailed an extended hemisection wherein a block of spinal cord was removed resulting in persistant motor deficits in the L. hindlimb. The surgery involved two, ipsilateral hemisections of the spinal cord at T_9, separated by 5 mm, with a midline commisurotomy between them. Preliminary studies revealed that technically the surgery was straight forward. We therefore initiated one study comprising 3 groups:

a. Sham-SCI (laminectomy without manipulation of the spinal cord)
b. SCI (administered vehicle beginning immediately after the wound for SCI surgery was closed)
c. SCI-Nan/Ts (administered nandrolone 0.75 mg/kg/wee and testosterone 2.8 mg/kg/day beginning immediately after closure of the wound for SCI surgery.

Animals were provided bladder care and bathing as needed until euthanasia at Day 56 after the surgeries.

Observation of the animals post-operatively revealed rapid development of automatic emptying of the bladder and excellent survival. Animals tended to rotate their pelvis such that the paralyzed side was dependent, which seemed to lead to irritation of the skin over the dependent side over hip and hindlimb. Of the 28 animals that underwent a hemisection, 4 died between days 5 and 7, 2 from peritonitis, 2 from bladder rupture. One was dropped because of a severe infection of the penis. Two were dropped because the hemisection was more extensive than desired as reflected by partial loss of function of the R leg, delayed development of automatic bladder emptying, and moving about by dragging the entire pelvis. Morphology of the spinal cord at the time of euthanasia confirmed that extensive damage was present in the spinal cord. At the time of sacrifice, casual inspection of the

In these studies we observed significant declines in the weight of the gastrocnemius, soleus, plantaris and EDL muscles of the paralyzed hindlimb indicating paralysis and resultant muscle atrophy (Figure 1). Body weights tended to decrease after SCI consistent with what we have

observed in other studies with male rats but there was a great deal of variability among animals with some gaining weight after SCI and others loosing significant body weight (Figure 1A and 1B).

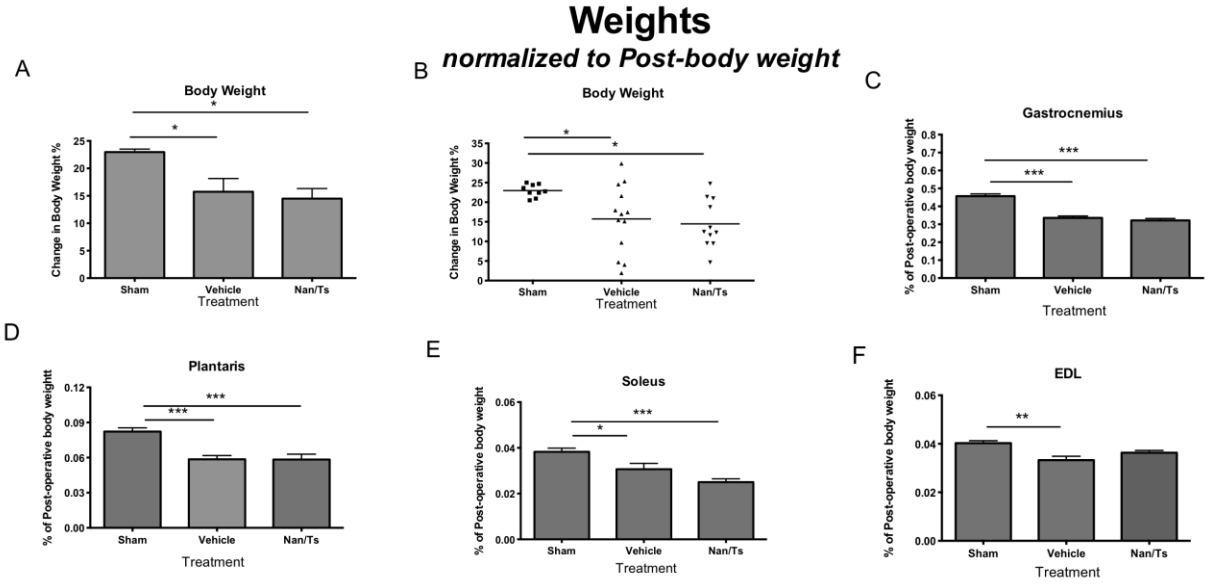


Figure 1. Body and Muscle Weights of rats at 56 days after SCI or Sham-SCI. Data are mean values ± SEM. Group sizes were: 10 Sham-SCI, 13 vehicle-treated SCI, and 11 nandrolone-testosterone-treated SCI. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.01$; ANOVA.

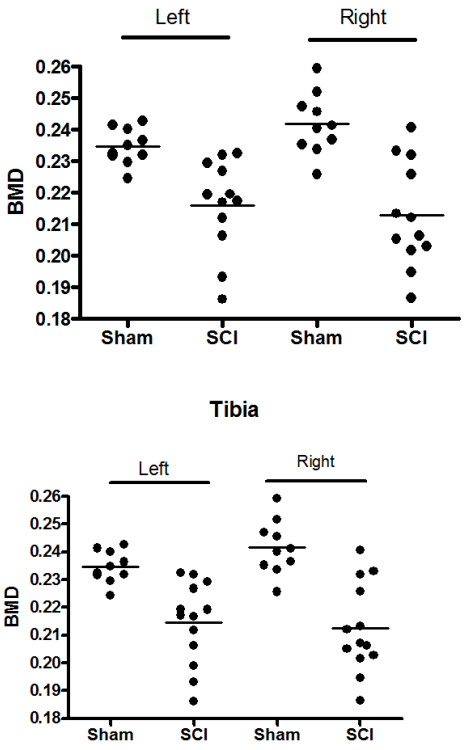


Figure 2. Effect of nandrolone on areal BMD of the distal femur and proximal tibia at 56 days after SCI induced by hemitransection at T9-10. N = 7 to 8 per group.

Bones were removed from the left and right hindlimbs of the animals and studied by dual energy X-ray absorbtiometry (DEXA) to evaluate bone loss in this SCI model and the effect of nandrolone to reduce such loss. While a decrease in the mean BMD of the bones of the left hindlimb was observed, interpretation of such change was limited due to tremendous variability of the decrease in bone mass (Figure 2). Also, unexpectedly, reductions in BMD of similar magnitude were observed for the right hindlimb (Figure 2).

<u>Conclusions: Task 1: Hemisection Model</u>

While survival was excellent and animal husbandry issues with bladder care were addressed by this model it has several important limitations that limited its use as a model for studies of bone loss after SCI. The first is the variability in body weights at the end of the period of observation which we attribute to variability in the degree of neurological impairment, and difficulty in precisely reproducing the hemisection across many animals. This variability in turn makes it difficult to resolve all but the largest effects of therapies, gene knockouts or knockins, or other manipulations.

Task 1: Alternative approach: A T4 complete transection in male rats.

It was reported that T_4 spinal cord transaction resulted in severe impairment of motor, sensory and autonomic function including that of the sympathetic nervous system [5]. It has been reported that automatic emptying of the bladder will develop within a few weeks and that the survival rate is normally about 90% (Stephen E. DiCarlo, personal communication). We were encouraged by reports that male rats with a complete T4 transection rapidly developed automatic emptying of the bladder to consider this model as an alternative to hemisection. The major advantage was a complete and easily reproduced neurological injury. Preliminary studies revealed that the surgery was feasible although complicated by the presence of a large vein which crossed the spinal cord under the arch of the third or fourth vertebral process. Initial losses during training surgeries were on the order of 50% but with some practice 90% of animals survived the surgery. Encouraged by this technical success we conducted pilot studies examining changes in muscle, bone and body weight after SCI. In our experience so far with 30 male rats with T4 transections, only 1 out of 30 animals died within the first 56 days, spontaneous voiding developed in all animals by 14 days, and all surviving animals were in good health at the time of euthanasia. Bone loss with the T4 transection after SCI is about 20% at the distal femur and proximal tibia at 56 days after SCI **(Figure 3)** which is similar to that for transection at T9-10 (please see the appended manuscript)**.** This model has been employed in our ancillary studies evaluating effects of an inactivating antibody against sclerostin described further below under Ancillary studies.

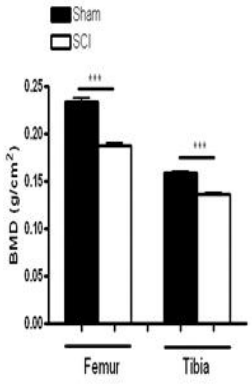


Figure 3. Bone loss in a rat model of T4 spinal cord transection. The effects of T4 spinal cord transection on BMD after 8 weeks are shown**.** Data are expressed as mean ± SEM. N = 13-15 per group. ***p<0.001 *versus* the indicated group by *t-test*.

Conclusions regarding the T4 complete transection model for studies of bone loss after SCI.

Our data thus far indicate that once the surgical technique is mastered the T4 complete transection provides a simple and reliable model of trabecular bone loss at the knee after an SCI. Our studies also demonstrate that this is a useful model for evaluating new therapeutics for their efficacy in reducing SCI-related bone loss. The ease of animal care with this model is a significant advantage for future research in this field.

Task 2. Characterization of beneficial effects of nandrolone in bone loss in SCI rats.

We have studied the anabolic effect of nandrolone in spinal cord injury (SCI) associated bone loss and a possible underlying mechanism of such effects. We completed a study about an anabolic effect of nandrolone in spinal cord injury associated bone loss in which we also identified a possible underlying mechanism **(Task 1, 2 and 5).** The manuscript describing findings of the study entitled "Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling" was recently accepted for publication in the Journal of Spinal Cord Medicine and is now in press **(A part of Task 8; Please refer to the appended manuscript, Page 19-25**) [2].

It is well appreciated that SCI causes severe bone loss and reduces circulating levels of testosterone [3, 4]. At present, there is no practical treatment to delay or prevent bone loss in individuals with SCI. We sought to test whether nandrolone, a synthetic anabolic steroid, reduced bone loss after SCI and the underlying mechanism of any osseous effects of nandrolone after this injury. For this purpose, male rats with complete transection of the spinal cord between the ninth and 10^{th} thoracic vertebra were administered nandrolone combined with a physiological replacement dose of testosterone or vehicle; drug administration was initiated on day 29 after SCI and was continued for 28 days (For detailed methods, please see **Appended manuscript, Page 20)**.

We first characterized the beneficial effects of nandrolone on bone loss in SCI rats **(Task 2).** We found that SCI reduced femoral and tibial BMD by approximately 20% at two months as determined by DXA scanning using a small animal bone densitometer (For detailed methods, please see the **Appended manuscript, Page 20)**. Bone loss was significantly attenuated by the administration of nandrolone **(Task 2; Appended manuscript Figure 1, Page 21)**. Our results demonstrate a beneficial role of nandrolone on bone loss after SCI. The findings suggest a potential mechanism whereby nandrolone modulates the differentiation and activity of ostoblasts and osteoclasts through increased Wnt signaling, at least in part, and associated favorable changes in the ratio of OPG to RANKL (**Appended manuscript Figure 4, Page 23**).

Tasks 3 and 4: Test the ability of animals with the novel model of SCI-related bone loss to survive for 6 months and the ability of nandrolone to stem bone loss in this model.

These studies were not initiated for two reasons. The completion of the studies for task 1 required more time and resources than anticipated, and findings acquired during the completion of Task 2 suggested several ancillary studies that promised to advance the field in alternative and important ways.

Task 5. Test whether nandrolone promotes net bone formation and bone cell differentiation and the role of calcineurin in this effect.

<u>Studies with primary cultures of osteoblasts obtained by differentiation of bone marrow stromal cells.</u>

Next, we tested the effects of nandrolone on bone cell differentiation after SCI **(Task 5).** We isolated bone marrow cells from femora and tibiae isolated from the rats studied for Task 2, above; specifically marrow cells were isolated at 56 days after SCI or a Sham-SCI. We performed *ex-vivo* cultures under conditions favoring differentiation into either osteoblasts or osteoclasts (For detailed methods, please see the **Appended manuscript, Page 20)**. We found that in ex-vivo cultured bone marrow cells, SCI increased mRNA levels of several osteoclast differentiation markers (tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor) and reduced mRNA levels of several osteoblast differentiation markers (Runx2, osteocalcin and sialoprotein (BSP)), as determined by quantitative real-time PCR (For detailed methods, please see the **Appended manuscript, Page 20)**. Most of these changes were significantly attenuated by nandrolone **(Task 5; Appended manuscript Figure 2, Page 22)**. Interestingly, osteoblast RANKL mRNA was increased more than 8 fold by SCI; nandrolone did not alter RANKL expression but increased expression of OPG, thereby favorably increasing the OPG/RANKL ratio **(Appended manuscript Figure 3, Page 22)**. Furthermore, SCI reduced mRNA levels of the Wnt signaling genes Wnt3a, LRP5, Fzd5 and ENC1 in osteoblasts whereas nandrolone significantly increased their expression **(Appended manuscript Figure 4, Page 23)**.

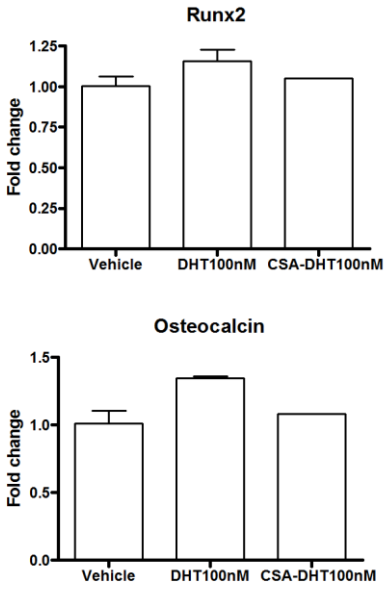


Figure 4. The mRNA levels for Runx2 and osteoclacin in cultured MC3T3 preosteoblasts. MC3T3 cells was treated with 100nM DHT or 100nM DHT plus 100ng/ml cyclosporin A (CSA) for 48 hours. Results are expressed as mean ± SEM of determinations.

Task 6. Test whether calcineurin is activated by nandrolone in cells; and Task 7. Test whether calcineurin is necessary for effects of nandrolone on bone cells.

To study mechanisms by which androgen affects differentiation of osteoblasts, we cultured preosteoblastsic MC3T3 cells in vitro. The MC3T3 cells was treated with 100nM DHT (an androgen) or co-treated 100nM DHT and 100ng/ml cyclosporin A (CSA, a calcineurin inhibitor) for 48 hours (Figure 4). The mRNA levels of markers of osteoblast differentiation, Runx2 and osteoclacin was examined. We found that DHT tends to increase mRNA levels of Runx2 and osteocalcin. The effect seems to be reduced by CSA, suggesting that androgens might influence the differentiation of osteoblasts via the calcineurin pathway. We are currently performing further experiments to extend these findings.

Task 8. Data Analysis, publication and grant applications.

Results of studies using the novel T4 transection model for studies of bone loss in male SCI rats (Task 1, Alternative approach) have been summarized in poster format (Poster #1, below, please see the attached abstract) as part of our studies of the effects of a neutralizing antibody against sclerostin on bone loss after SCI. Most data regarding the phenotype of bone in this model has been collected, and a manuscript describing the results of these data is anticipated during the Winter of 2013-14.

Results of our studies of the effects of nandrolone on bone mass after SCI (Task 2) have been analyzed and reported in a manuscript now in press in J. Spinal Cord Medicine (please see the attached manuscript); this manuscript also reports results of our findings from studies of gene expression changes in cultured osteoblasts derived from bone marrow stromal cells (Task 5).

Our ancillary studies of the effects of a knockout of the sclerostin gene on bone loss after SCI will be reported in preliminary form the aforementioned poster to be presented at the annual meeting of the ASBMR in October. We are continuing the final analyses of bone for the samples from these studies and we anticipate a manuscript to be submitted during the Winter of 2013-14.

Preliminary data from our studies for Tasks 1 and Task 2 have supported a successful application for a VA Merit Review award entitled “ES and Androgens in Bone Loss after SCI:

Synergistic Effects and Mechanisms"" in which we will examine the effects of reloading of bone by electrical stimulation of neuromuscular injury on bone loss after SCI when administered alone or combined with nandrolone.

Preliminary studies from Task 1 and our Ancillary studies have been included to support an application to the VA Merit Review program entitled "Sclerostin Antagonism and the Osteocyte's Role: Prevention of Bone Loss after SCI" for support for studies of the effects of neutralizing antibodies to prevent or reverse bone loss after SCI when initiated either at the time of the SCI or one month later. This application scored very well during the first review and we are hopeful that the revised proposal will be sufficiently improved to be funded.

The T4 transection developed under Task 1 has also been proposed for studies that will evaluate the effects of an antagonist of myostatin and other Activin receptor IIB ligands on the loss of muscle and bone after SCI in an application to the VA Merit Award program entitled "Beneficial effects of soluble Activin receptor IIB-mFc after SCI".

Ancillary Studies.

During the first year and the early second year of the funding period, we discovered an unexpected mechanism by which nandrolone reduced bone loss in our animal model of SCI, involving regulation of Wnt signaling. We thought this was an important finding because the Wnt pathway has been reported to have an anabolic action on bone by regulating osteoblast function. Indeed it has been reported that the regulation of Wnt signaling in bone by Wnt inhibitors (e.g., sclerostin, DKK1 and sFRPs) is crucial in the pathogenesis of disuse osteopenia [7-10]. We reasoned that anti-sclerostin antibody may be anticipated to be an attractive candidate for reducing and/or reversing the rapid and extensive bone loss resulting from SCI. Thus we adjusted our priorities and efforts to pursue this interesting research direction.

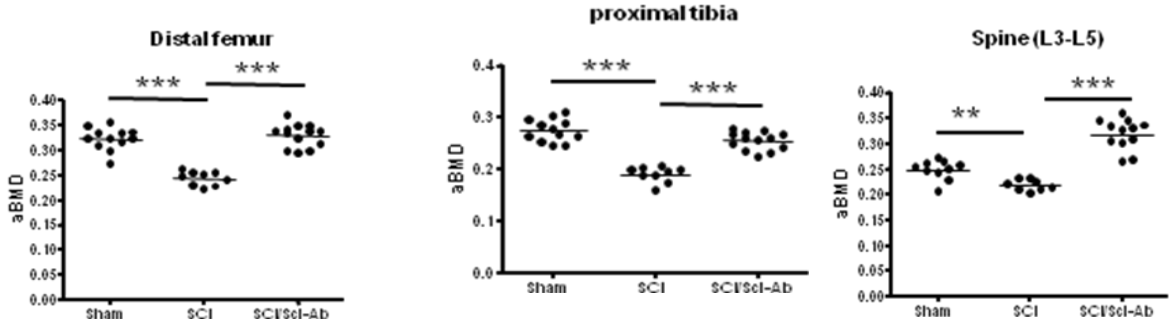
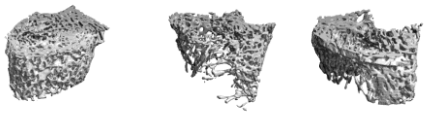


Figure 5. Effects of Scl-Ab on Bone Mass. The effects of 8 weeks of Scl-Ab begun at 1 week after SCI are shown for areal BMD (aBMD) at distal femur, proximal tibia and spine (L3-5) as examined by DXA. Data are expressed as mean ± SEM. N = 8 to 12 per group. ** p<0.01 and ***p<0.001 *versus* the indicated group.



Sham **SCI** **SCI/Scl-Ab**

Figure 6. Scl-Ab also significantly restored the deteriorated bone micro-architecture. The effects of 8 weeks of Scl-Ab begun at 1 week after SCI are shown for trabucular microarchitecture by micro-CT.

In collaboration with Amgen, we have conducted a pilot study to evaluate the efficacy of an anti-sclerostin antibody to prevent bone loss after acute SCI in rats. We were excited to find that when started at 7 days after injury, administered once a week (i.p. 25mg/kg) and continued for 2 months, the Scl-Ab almost completely prevented SCI-induced bone loss in the femur, tibia and lumbar spine (**Figure 5**)

acutely after injury. Scl-Ab also largely preserved trabacular microarchitecture as assessed by microCT of the fumur (**Figure 6**). In parallel, Scl-Ab increased osteoblastogenesis (**Figure 7**) and decreased osteoclastogenesis of ex vivo cultured bone marrow stem cells (**Figure 8**). This evidence established anti-sclerostin antibodies as the first agent to demonstrate efficacy to completely block the marked bone loss after acute SCI; this also suggested that the Scl-Ab has both potent anabolic and anti-resorptive effects in our rat model of acute SCI.

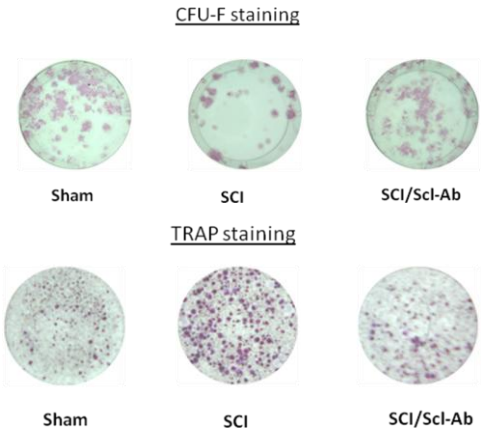


Figure 7. Scl-Ab increased osteoblastogenesis. The photomicrographs show the effects of 8 weeks of Scl-Ab begun at 1 week after SCI on cultures of marrow-derived osteoblasts that were stained at 10 days of culture for alkaline phosphatase (CFU-F).

Figure 8. Scl-Ab reduced osteoclastogenesis. The photomicrographs show the effects of 8 weeks of Scl-Ab begun at 1 week after SCI on numbers of TRAP-positive multinucleated cell derived from bone marrow (osteoclasts).

Key Research Accomplishments

Our results thus far have demonstrated a beneficial role of nandrolone on bone loss after SCI. The findings suggest a potential mechanism whereby nandrolone modulates the differentiation and activity of ostoblasts and osteoclasts through increased Wnt signaling, in part, and associated favorable changes in the ratio of OPG to RANKL.

Our recent findings also suggested that Scl-Ab almost completely prevented SCI-induced bone loss and preserved trabecular bone microarchitecture acutely after injury. Scl-Ab also increased osteoblastogenesis and decreased osteoclastogenesis of ex-vivo cultured bone marrow stem cells

Reportable Outcomes

Task 1 and Ancillary Studies

A T4 complete transection of the spinal cord provided a model of bone loss after SCI in male rats with excellent survival (> 90% of animals survived 56 days) and substantial bone loss of a magnitude to easily examine the ability of interventions to reduce such loss. Characteristics of the model will be reported in poster format (poster 1, below) and we anticipate a manuscript providing more detail to be submitted during the Winter of 2013-14; the title and author list are indicated below (Manuscript 1, below). Results of studies examining the effects of a knockout of sclerostin on bone loss after SCI in mice have been completed and will be reported in part in Poster 1, below, as well as in Manuscript 2, below.

Poster 1.

Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling. *Li Sun, Jiangping Pan, Yuanzhen Peng, Yong Wu, Jianghua Li, Xuan Liu, Yiwen Qin, William A. Bauman, Christopher Cardozo, Mone Zaidi and Weiping Qin* **(See appended Abstract)**.

Oral Poster Presentation.

American Society for Bone and Mineral Research Annual Meeting, Baltimore, MD (Oct 4-7, 2013) **(See appended Abstract)**.

Manuscript 1.

Therapeutic Potential of Sclerostin Antibody in Extreme Disuse-induced Bone Loss after Spinal Cord Injury: a Preclinical Animal Study.

Weiping Qin[1,2], Xiaodong Li[4], Jay Cao[5], Lauren Collier[1], Yuanzhen Peng[1], Jiliang Li[5],Hua Zhu Ke[4], William A. Bauman[1,2,3], and Christopher Cardozo[1,2,3].

Manuscript 2.

Sclerostin is necessary for loss of trabecular bone mass after spinal cord injury.

Weiping Qin[1,2], Xiaodong Li[4], Jay Cao[5], Lauren Collier[1], Yuanzhen Peng[1], Jiliang Li[5],Hua Zhu Ke[4], William A. Bauman[1,2,3], and Christopher Cardozo[1,2,3].

Task 2 and Task 5.

Nandrolone reduced bone loss at 56 days after SCI when initiated 28 days after SCI. In this model, nandrolone was initiated after the majority of bone loss had occurred suggesting that nandrolone may have reversed at least some of the bone loss that had occurred.

Studies of gene expression in ex-vivo cultured osteoblasts obtained by differentiation of bone marrow stromal cells from the tibia and fibula of animals with SCI or a Sham SCI indicated that one action of nandrolone was to partially reverse the downregulation of genes expressed in osteoblasts including Runx2, osteocalcin and bone sialoprotein. Finally, whereas expression of Wnt signaling genes Wnt3a, LRP5, Fzd5, and of the Wnt-responsive gene ectodermal-neural cortex 1 (ENC1) were reduced by SCI, expression of each of these genes was increased by nandrolone indicating both an increased expression of Wnt signaling components and a net increase in Wnt signaling. These results have been reported in a manuscript that is now available online and which is in press:

Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling. *Li Sun, Jiangping Pan, Yuanzhen Peng, Yong Wu, Jianghua Li, Xuan Liu, Yiwen Qin, William A. Bauman, Christopher Cardozo, Mone Zaidi and Weiping Qin.*
Journal of Spinal Cord Medicine (in press) **(See appended manuscript)**.

Conclusion

Our preclinical study in rat model of SCI suggested that nandrolone is an effective agent to significantly reduce bone loss after SCI. Thus, our findings suggest a potential clinical use for nandrolone or other anabolic steroids to reduce bone loss after SCI.

In addition, our preliminary established anti-sclerostin antibodies as the first agent to demonstrate efficacy to completely block the marked bone loss after acute SCI through potent anabolic and anti-resorptive actions and support clinical investigations with this class of agent in acutely after SCI to preserve bone mass..

Future studies

Because our data established anti-sclerostin antibodies as a very potent anabolic and anti-resorptive agent that is capable of completely blocking the marked bone loss after acute SCI, in future studies we plan:

1) To evaluate the efficacy of anti-sclerostin antibody or nandrolone to reverse bone loss when such treatment is initiated one or more months after SCI, e.g., during the sub-acute or chronic period of SCI when loss of bone exceeds 20% of BMD and 50% of trabecular bone at the distal femur and proximal tibia.

2) To determine whether the combination of anti-sclerostin antibody and nandrolone act synergistically to reduce SCI-induced bone loss.

3) To test whether nandrolone promotes bone cell differentiation and net bone formation, and to determine the role of calcineurin on these effects, as originally proposed (**Task 5, 6** and **7**).

Bibliography of all publications

1. Li Sun, Jiangping Pan, Yuanzhen Peng, Yong Wu, Jianghua Li, Xuan Liu, Yiwen Qin, William A. Bauman, Christopher Cardozo, Mone Zaidi and Weiping Qin. "Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling". *The journal of Spinal Cord Medicine*. In press. **(See appended manuscript)**.

Personnel (not salaries) receiving pay from the research effort

Lauren Collier

Yong Wu

References:

1. Primeaux, S.D., M. Tong, and G.M. Holmes, *Effects of chronic spinal cord injury on body weight and body composition in rats fed a standard chow diet.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(3): p. R1102-9.
2. Qin, W., Peng, Y., Liu, X., Wu, Y., Pan, J., Bauman, W.A., Zaidi, M., Sun, L., Cardozo, C.P., *Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling. Journal of Spinal Cord Medicine. In Press.*
3. Qin, W., W.A. Bauman, and C. Cardozo, *Bone and muscle loss after spinal cord injury: organ interactions.* Ann N Y Acad Sci. **1211**: p. 66-84.
4. Qin, W., W.A. Bauman, and C.P. Cardozo, *Evolving concepts in neurogenic osteoporosis.* Curr Osteoporos Rep. **8**(4): p. 212-8.
5. Kalincik, T., et al., *Selected changes in spinal cord morphology after T4 transection and olfactory ensheathing cell transplantation.* Auton Neurosci. **158**(1-2): p. 31-8.
6. Cuscito, C., et al., *Adrenergic stimulation decreases osteoblast oxytocin synthesis.* Ann N Y Acad Sci. **1237**: p. 53-7.
7. Srinivasan, S., et al., *Role of mitochondrial reactive oxygen species in osteoclast differentiation.* Ann N Y Acad Sci. **1192**: p. 245-52.

Meeting abstracts

1. The 2012 Military Health System Research Symposium. Title: Anabolic Steroids Reduce Spinal Cord Injury-Related Bone Loss in Rats Associated with Increased Wnt Signaling.

PURPOSE/AIMS: Spinal cord injury (SCI) causes severe bone loss. At present, there is no practical treatment to delay or prevent bone loss in individuals with motor-complete SCI. Hypogonadism is common in men after SCI and may exacerbate bone loss. The anabolic steroid nandrolone reduces bone loss due to microgravity or nerve transection. The purpose of this study was to determine whether nandrolone reduced bone loss after SCI and, if so, to explore the mechanisms of nandrolone action.

DESIGN: Male rats with complete transection of the spinal cord were administered nandrolone combined with a physiological replacement dose of testosterone, or vehicle, beginning on day 29 after SCI and continued for 28 days.

POPULATION/SAMPLE STUDIED: Bone (Femur and tibia) and bone marrow cells.

METHOD(S): Areal BMD measurements were performed by using a small animal bone densitometer (Piximus, Fitchburg WI). E*x vivo* osteoclastogenesis and osteoblastogenesis assays was conducted according to the procedures that have previously described [1]. qPCR was performed according to the procedures that have previously described [2].

DATA ANALYSIS: The statistical significance of differences among means was tested using one-way ANOVA, and a Newman-Keuls test post hoc to determine the significance of differences between individual pairs of means.

FINDINGS: SCI reduced distal femoral and proximal tibial bone mineral density (BMD) by 25% and 16%, respectively, at 56 days. This bone loss was attenuated by nandrolone. In ex-vivo osteoclasts cultures from bone marrow Hematopoietic cells, SCI increased mRNA levels for tartrate resistant acid phosphatase (TRAP) and calcitonin receptor; nandrolone normalized expression levels of these transcripts. In ex-vivo osteoblast cultures derived from bone marrow stromal cells, SCI increased RANKL but did not alter osteoprotegerin (OPG) mRNA expression; nandrolone increased expression of OPG and OPG/RANKL ratio. SCI reduced mRNA levels of Wnt signaling related genes Wnt3a, LRP5, Fzd5, Tcf7 and ENC1 in osteoblasts, whereas nandrolone increased expression of these genes.

CONCLUSIONS/RECOMMENDATIONS: Our results demonstrate that nandrolone reduces bone loss after SCI. A potential mechanism is suggested by our findings wherein nandrolone modulates genes for differentiation and activity of osteoclasts and osteoblasts, at least in part, through the activation of Wnt signaling.

IMPLICATIONS: The major finding of the present study is that nandrolone reduced bone loss in a rat model of hindlimb paralysis caused by a complete SCI. This finding is of potential clinical relevance because bone loss in individuals with motor complete SCI is rapid, severe, and refractory to bisphosphonates.

FROM/TO TIME PERIOD OF STUDY: 2010.9-2012.3

FUNDING: Department of Defense grant SC090504

REFERENCE:

1. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y *et al. Cell* 2003; **115**(2)**:** 151-62.

2. Qin W, Pan J, Wu Y, Bauman WA, Cardozo C. *BBRC*. 2010 Dec 17;403(3-4):473-8. Epub 2010 Nov 19.

2. **ASBMR 2013 Abstract (**ID: A13010173)

Therapeutic Potential of Sclerostin Antibody in Extreme Disuse-induced Bone Loss after Spinal Cord Injury: a Preclinical Study.

Weiping Qin[1,2], Xiaodong Li[4], Jay Cao[5], Lauren Collier[1], Yuanzhen Peng[1], Jiliang Li[5],Hua Zhu Ke[4], William A. Bauman[1,2,3], and Christopher Cardozo[1,2,3].

[1] National Center of Excellence for the Medical Consequences of SCI, James J. Peters VA Medical Center, Bronx, NY; [2] Departments of Medicine and Rehabilitation Medicine, Mount Sinai School of Medicine, New York, NY, [4] Amgen Inc., Thousand Oaks, CA; [5] USDA ARS Human Nutrition Research Center, Grand Forks, ND; [6] Indiana University Purdue University, Indianapolis, IN.

The rapid and extensive sublesional bone loss after spinal cord injury (SCI) is a difficult medical problem that has been refractory to available interventions tested to date (1-3). Inhibition of sclerostin by sclerostin monoclonal antibody (Scl-Ab) has been shown to increase bone formation and improve bone mass and strength in animal models of bone loss due to estrogen deficiency and immobilization (4). Scl-Ab also increased bone mineral density (BMD) in healthy men and postmenopausal women (4). In addition, sclerostin has been proposed as a potential mediator of mechanotransduction (5). Therefore, we hypothesized that sclerostin could be responsible for the bone loss due to SCI and, if so, that sclerostin inhibition with Scl-Ab could provide a hopeful solution for this challenging medical problem. To test our hypotheses, we first performed complete spinal cord transection in sclerostin knockout (SOST $^{-/-}$) mice and wild type (WT) controls. Eight weeks after SCI, significant bone loss was observed at the distal femur and proximal tibia in WT mice. However, no bone loss was observed in SOST $^{-/-}$ mice. These findings suggest that sclerostin mediates sublesional bone loss after SCI. To explore the therapeutic potential of sclerostin inhibition in SCI, male Wistar rats underwent complete spinal cord transection; 7 days after SCI, the rats were treated with Scl-Ab at 25 mg/kg/week or vehicle for 7 weeks. SCI resulted in significant decreases in BMD (-25%) and trabecular bone volume (-66%) at the distal femur. Scl-Ab completely prevented the loss of BMD and trabecular bone volume. Additionally, in SCI rats administered Scl-Ab, Tb.Th was increased to levels above values for non-SCI controls, and Tb.N tended to be higher than SCI controls. Preliminary histomorphometric analysis demonstrated that Scl-Ab increased trabecular bone formation. In cultures of bone marrow cells, SCI increased the number of TRAP^{+} multinucleated cells as well as mRNA levels of osteoclast differentiation markers, and reduced the number of osteoblasts and mRNA levels of the osteoblast differentiation markers. None of these deleterious changes were observed in the Scl-Ab-treated group. In summary, our findings indicate that Scl-Ab fully prevented sublesional loss of BMD and metaphysis trabecular bone volume by increasing bone formation and decreasing bone resorption, strongly suggesting that Scl-Ab may represent a promising novel approach to mitigate the striking bone loss after SCI.

Sources of Research Support: This work was supported by the Department of Defense grant SC090504 to WQ, the Veterans Health Administration, Rehabilitation Research and Development Service (grants B9212-C *to WAB). Amgen Inc. and UCB Pharmap provided Scl-Ab.*

References:

1. Qin W, Bauman WA, Cardozo C. Bone and muscle loss after spinal cord injury: organ interactions. *Ann N Y Acad Sci*. 2010, 1211:66-84.

2. Qin W, Bauman WA, Cardozo CP. Evolving concepts in neurogenic osteoporosis. *Curr Osteoporos Rep*. 2010, 8:212-218.

3. Jiang SD, Jiang LS, Dai L. Mechanisms of osteoporosis in spinal cord injury. *Clin Endocrinol (Oxf.)* 2006, 65:555-565.

4. Ke HZ, Richards WG, Li X, Ominsky MS. Sclerostin and Dickkopf-1 as therapeutic targets in bone diseases. *Endocrine Review,* 2012. 33(5):747-83.

5. Bonewald, L.F. and M.L. Johnson, Osteocytes, mechanosensing and Wnt signaling. *Bone.* 2008. 42(4): 606-15.

Appendices

1. The accepted manuscript entitled “Anabolic steroids reduce bone loss due from spinal cord injury associated with increased Wnt signaling”.

Research article

Anabolic steroids reduce spinal cord injury-related bone loss in rats associated with increased Wnt signaling

Li Sun[1], Jiangping Pan[2], Yuanzhen Peng[1], Yong Wu[2], Jianghua Li[1], Xuan Liu[1], Yiwen Qin[2], William A. Bauman[2,3,4], Christopher Cardozo[2,3,4], Mone Zaidi[1], Weiping Qin[2,3]

[1]Mount Sinai Bone Program, Mount Sinai School of Medicine, New York, NY, USA, [2]Center of Excellence for the Medical Consequences of SCI, James J. Peters VA Medical Center, Bronx, NY, USA, [3]Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA, [4]Department of Rehabilitation Medicine, Mount Sinai School of Medicine, New York, NY, USA

Background: Spinal cord injury (SCI) causes severe bone loss. At present, there is no practical treatment to delay or prevent bone loss in individuals with motor-complete SCI. Hypogonadism is common in men after SCI and may exacerbate bone loss. The anabolic steroid nandrolone reduces bone loss due to microgravity or nerve transection.

Objective: To determine whether nandrolone reduced bone loss after SCI and, if so, to explore the mechanisms of nandrolone action.

Methods: Male rats with complete transection of the spinal cord were administered nandrolone combined with a physiological replacement dose of testosterone, or vehicle, beginning on day 29 after SCI and continued for 28 days.

Results: SCI reduced distal femoral and proximal tibial bone mineral density (BMD) by 25 and 16%, respectively, at 56 days. This bone loss was attenuated by nandrolone. In *ex vivo* osteoclasts cultures, SCI increased mRNA levels for tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor; nandrolone-normalized expression levels of these transcripts. In *ex vivo* osteoblast cultures, SCI increased receptor activator of NF-kB ligand (RANKL) mRNA levels but did not alter osteoprotegerin (OPG) mRNA expression; nandrolone-increased expression of OPG and OPG/RANKL ratio. SCI reduced mRNA levels of Wnt signaling-related genes Wnt3a, low-density lipoprotein receptor-related protein 5 (LRP5), Fzd5, Tcf7, and ectodermal-neural cortex 1 (ENC1) in osteoblasts, whereas nandrolone increased expression of each of these genes.

Conclusions: The results demonstrate that nandrolone reduces bone loss after SCI. A potential mechanism is suggested by our findings wherein nandrolone modulates genes for differentiation and activity of osteoclasts and osteoblasts, at least in part, through the activation of Wnt signaling.

Keywords: Spinal cord injuries, Nandrolone, Androgens, Hypogonadism, Bone loss, Wnt signaling

Introduction

Spinal cord injury (SCI) causes unloading of skeletal regions immobilized by paralysis and extensive loss of sublesional bone.[1,2] The regions most affected by SCI are the distal femur and proximal tibia, where decreases in bone mineral density (BMD) may exceed 50%.[1,2] Such loss results largely from increased resorption of bone mediated through the increase of the numbers and activity of osteoclasts.[1,2] Bone resorption is stimulated by cells of the osteoblast lineage by the release of receptor activator of NF-kB ligand (RANKL), which stimulates differentiation and activity of osteoclasts.[3] RANKL is responsible for a great deal of the bone loss resulting from immobilization such as that occurring after SCI.[3] Cells of the osteoblast lineage also release osteoprotegerin (OPG), which inhibits RANKL.[3] Osteocytes, which are derived from osteoblasts after

Correspondence to: Yiwen Qin, James J. Peters VA Medical Center, 130 West Kingsbridge Road, Bronx, NY 10468, USA.
Email: Weiping.qin@mssm.edu

DOI 10.1179/2045772312Y.0000000020

they become encased in bone, are the major source of RANKL in unloaded bone.[4]

The Wnt/β-catenin pathway is an important determinant of bone mass.[3,5] Canonical Wnt signaling occurs when Wnt signaling proteins such as Wnt3a bind to and activate a receptor complex composed of a frizzled receptor, of which there are 10, and either low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6.[3,5] Frizzled receptors then activate β-catenin which translocates to the nucleus.[3,5] Much of the signaling through β-catenin involves its binding to members of the TCF/LEF family of transcription factors, such as Tcf7 (also known as Tcf1).[3,5] Wnt signaling is coupled to mechanical loading of bone by alterations in osteocyte expression of the SOST gene, which encodes the Wnt inhibitor sclerostin.[6] Canonical Wnt signaling promotes osteoblastic differentiation of mesenchymal precursor cells through upregulation of Runx2.[7] Wnts stimulate osteoblasts to release OPG and to reduce expression of RANKL,[5,8] thereby reducing osteoclastogenesis and osteoclast activation.

Reduced levels of testosterone are common in men after SCI.[1,2] Hypogonadism results in high-turnover bone loss,[9,10] and thus may accelerate SCI-related bone loss. Interestingly, androgens, including nandrolone and testosterone, reduce bone loss after unweighting or nerve transaction.[11,12] Whether androgens prevent SCI-induced bone loss in animal models or humans is not known. Little is known about the molecular mechanisms by which androgens preserve bone in states of unloading or paralysis. Because androgens promote Wnt-dependent differentiation of cultured MC3T3 preosteoblasts,[13] one possibility is that androgens alter Wnt signaling in bone cells.

In this study, we tested whether nandrolone could reduce bone loss occurring in rats after transection of the mid-thoracic spinal cord. We also used *ex vivo* cultures of bone marrow cells to test the effects of nandrolone on expression levels of mRNA-encoding molecules necessary for osteoblast and osteoclast differentiation and activity, and of genes related to Wnt signaling.

Methods

Animals, spinal cord transaction, and drug administration

All animal studies were approved by the Institutional Animal Care and Use Committee at the James J. Peters VA Medical Center. As approximately 80% of individuals with SCI are male,[14] these studies were conducted using male rats. Because we found that administration of nandrolone during the second month after nerve transection reduced bone loss,[12] in the current study, administration of nandrolone was begun 29 days after SCI, and the animals were euthanized at day 56. Male Wistar rats aged 8 weeks were obtained from Taconic Farms (Hudson, NY, USA). One week later, the animals were anesthetized by inhalation of isoflurane and the spinal cord was transected at the interspace between the 9th and 10th vertebral bodies. Urine was voided at least three times daily; reflex voiding was not observed. Baytril was administered for the first 3–5 days postoperatively, and then as indicated for cloudy or bloody urine or for overt wound infection. Sham-transected (Sham-SCI) animals ($N = 7$) received an identical surgery, including a laminectomy, except that the spinal cord was not cut. Twenty-nine days after SCI rats were anesthetized with isoflurane, then randomly assigned to undergo implantation of Alzet pumps that infused either vehicle (SCI-vehicle; propylene glycol; Sigma, St Louis, MO, USA; $N = 8$) or nandrolone (SCI-Nandrolone; 0.75 mg/kg/week, Sigma; $N = 8$) plus testosterone (2.8 mg/kg/day; Spectrum Chemical Co., Gardena, CA, USA). Testosterone was administered together with nandrolone to control for effects of nandrolone to reduce circulating levels of testosterone due to feedback inhibition of central release of gonadotropins. The treatment of nandrolone plus testosterone is referred to henceforth in the text as "nandrolone."

Following spinal cord transection, the animals lost all voluntary movement of the knee and ankle joint, as well as most voluntary movement of the hip joint. There was no apparent difference among the groups in activity or movements of these joints or spasticity at any time during the 56 days after SCI. The animals were euthanized 56 days after SCI; at this time nandrolone had been administered for 28 days. Euthanasia was performed by transection of the aorta after anesthesia with isoflurane. The tibia, fibula, and femur were removed with the knee joint intact after carefully separating bone from muscle and connective tissue.

Bone densitometry

Areal BMD measurements were performed by using a small animal bone densitometer (Piximus, Fitchburg, WI, USA). The instrument was calibrated using a phantom following the manufacturer's recommendations. The precision for BMD measurements (coefficients of variation) is approximately 1.5% for the regions of interest (ROI). The femur and tibia were placed on a tray with the knee forming a 45° angle. Distal femur and proximal tibia were selected as ROI.

Ex vivo *osteoclastogenesis and osteoblastogenesis assays*

Procedures were adapted from those we have previously described.[15] To study osteoclast formation, bone marrow cells were isolated from the femora and tibiae in α-MEM. Marrow cells were rinsed and resuspended in α-MEM then seeded into wells using an equal number of cells in each well, and were cultured for 2 days in α-MEM supplemented with human macrophage colony-stimulating factor (M-CSF; 5 ng/ml). The non-adherent cells were collected and purified by Ficoll-Plus (Amersham Pharmacia Biotech Inc., Arlington Height, IL, USA) then seeded into wells, again with an equal number of cells per plate, and incubated in α-MEM containing M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 4–6 days. To study osteoblast formation, equal numbers of bone marrow cells were seeded into wells and cultured in α-MEM supplemented with 15% preselected FCS (Hyclone, Logan, UT, USA) and ascorbic acid-2-phosphate (1 mM). Incubation was continued until day 28.

Quantitative polymerase chain reaction

Total RNA was extracted from cultured bone marrow cells and used to synthesize cDNA libraries that were used to measure mRNA levels by real-time quantitative polymerase chain reaction (qPCR) using TaqMan 2X PCR buffer. These measurements were performed using Assay on Demand probe sets obtained from Applied Biosystems (Forster City, CA, USA). qPCRs were performed in triplicate following the manufacturer's recommended procedures. The average of the crossing points for the replicates was used in subsequent calculations. Changes in mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with levels of 18S ribosomal RNA serving as the internal control.

Statistics

The data are expressed as mean ± SEM. The number of animals (N) is provided in the legend of each figure. The statistical significance of differences among means was tested using one-way analysis of variance and a Newman–Keuls test *post hoc* to determine the significance of differences between individual pairs of means. A P value of 0.05 was used as the cut-off for significance of differences. Statistical calculations were performed using Prism 4.0c (Graphpad Software, La Jolla, CA, USA).

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Results

At 56 days after SCI, BMD for the SCI-vehicle group at the distal femur and proximal tibia were reduced ($P < 0.05$) compared with the Sham-SCI group by 25 and 16%, respectively (Figs. 1A and B). BMD in these regions was increased in the SCI-nandrolone group ($P < 0.05$) compared with the SCI-vehicle group by approximately 30%, although the values for BMD in the SCI-nandrolone group remained below the values observed for the Sham-SCI group (Figs. 1A and B).

Using total RNA from *ex vivo* cultures of bone marrow cell-derived osteoclasts, the expression of mRNA transcripts encoding two markers of osteoclast differentiation, tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor was examined. At 56 days after SCI, an elevation (two-fold; $P < 0.05$) of each transcript was observed compared with the Sham-SCI group (Fig. 2A). The levels of these mRNAs were reduced ($P < 0.01$) for the SCI-nandrolone group compared with the SCI-vehicle group to values that were not significantly different from the Sham-SCI group (Fig. 2A).

The mRNA levels for the osteoblast differentiation marker Runx2, and for the osteoblast-derived bone proteins osteocalcin and bone sialoprotein (BSP) were also assessed in *ex vivo* cultures of bone marrow-derived osteoblasts. The levels of all three mRNAs were markedly reduced ($P < 0.001$) for the SCI-vehicle group compared with the Sham-SCI group (Fig. 2B). The levels for Runx2 and osteocalcin were increased ($P < 0.05$) by 30 and 250%, respectively, for the SCI-nandrolone group compared with the SCI-vehicle group, although these

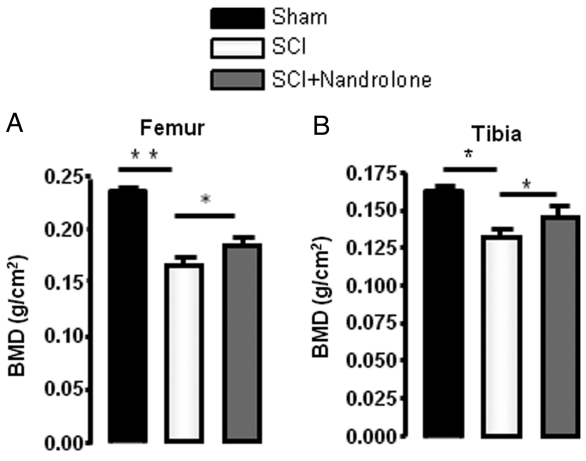


Figure 1 Effect of nandrolone on areal BMD of (A) the distal femur and (B) proximal tibia at 56 days after SCI. $N = 7 - 8$ per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$ and **$P < 0.01$ versus the indicated group.

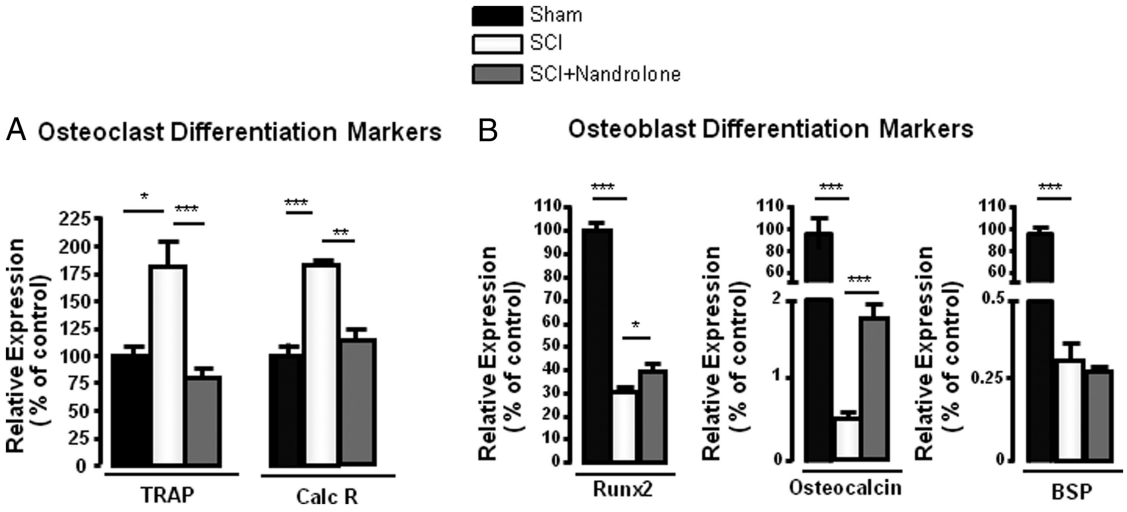


Figure 2 mRNA levels of differentiation markers in *ex vivo* cultured osteoclasts and osteoblasts derived from bone marrow cells from femur and tibia. (A) Levels in osteoclasts of mRNAs for TRAP and calcitonin receptor (Calc R). (B) Levels in osteoblasts of mRNAs for Runx2, osteocalcin, and BSP. Results are expressed as mean ± SEM for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the indicated group.

levels remained greatly reduced compared with the Sham-SCI group (Fig. 2B).

The levels for RANKL mRNA in *ex vivo* cultures of bone marrow-derived osteoblasts were markedly ($P < 0.001$) increased for the SCI-vehicle group compared with the Sham-SCI group; mRNA levels for OPG were not significantly different between these groups. Thus, SCI led to a marked decrease ($P < 0.05$) in the OPG/RANKL ratio (Fig. 3). Expression of OPG was increased ($P < 0.05$) in the SCI-nandrolone group compared with the SCI-vehicle group, without significant changes in RANKL mRNA levels (Figs. 3A and B), resulting in an increase ($P < 0.05$) in the OPG/RANKL ratio (Fig. 3C).

To evaluate changes in Wnt signaling, mRNA levels for Wnt3a, LRP5, Fzd5, and the Wnt-responsive gene ectodermal-neural cortex 1 (ENC1)[16] were examined. Expression of one transcription factor from the TCF/LEF family, Tcf7, was also examined. The levels for all of these transcripts were greatly reduced ($P < 0.05$) in the SCI-vehicle group compared with the Sham-SCI group (Figs. 4A–E). The levels of each of these mRNAs were increased ($P < 0.05$) for the SCI-nandrolone group compared with the SCI-vehicle group (Figs. 4A–E). More importantly, the expression level of Wnt3a for the SCI-nandrolone group was greater than that of the Sham-SCI group. The effects of nandrolone on expression levels of Tcf7, LRP5, and Fzd5 were

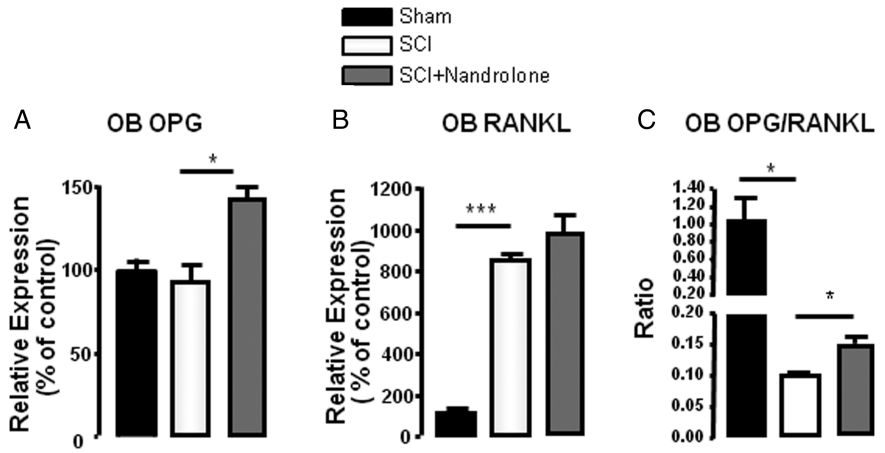


Figure 3 mRNA levels for OPG, RANKL and OPG to RANKL ratio in *ex vivo*-cultured osteoblasts. (A–C) Results are expressed as mean ± SEM of determinations for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls *post hoc* test. *$P < 0.05$, ***$P < 0.001$ versus the indicated group. OB, osteoblast.

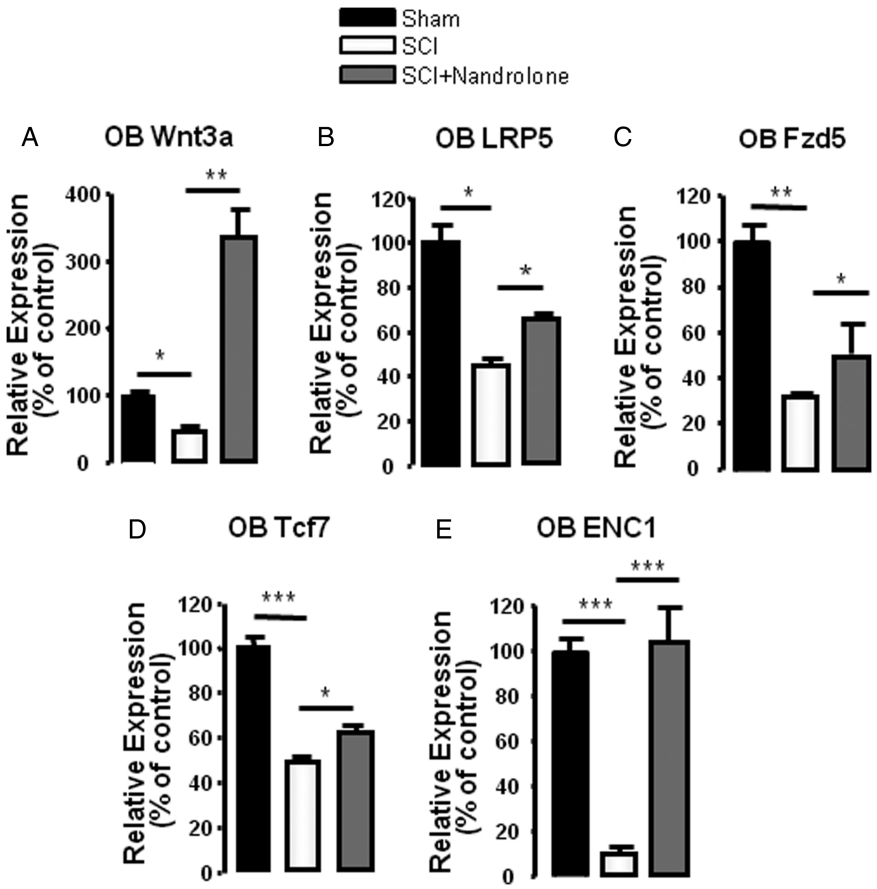


Figure 4 Effects of nandrolone on Wnt signaling. (A–E) Expression of Wnt3a, LRP5, Fzd5, Tcf7, or ENC1 mRNAs in *ex vivo* cultures of osteoblasts. Results are expressed as mean ± SEM of determinations for three to four animals per group. Significance of differences was determined using one-way analysis of variance with a Newman–Keuls test *post hoc*. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the indicated group.

less dramatic; the levels for the SCI-nandrolone group remained lower than those for the Sham-SCI group (Figs. 4B–D), while the levels of ENC1 were same in the SCI-Sham and SCI-vehicle groups (Fig. 4E).

Discussion

Bone loss and alterations in gene expression after SCI in rats

Substantial loss of bone mineral content was found for the distal femur and proximal tibia of rats at 56 days after SCI; these are the two skeletal regions most greatly affected by SCIs in humans.[1,2] We found increased expression of two osteoclast markers in *ex vivo* osteoclast cultures from SCI rats, suggesting activation of these cells. We also observed marked increase in expression of RANKL, which stimulates osteoclast formation from marrow progenitor cells,[3] in *ex vivo* cultures of osteoblasts from SCI rats, consistent with increased RANKL expression by osteocytes in other models of bone unloading.[4]

Wnt signaling, a critical determinant of bone mass, exerts anabolic effects on osteoblasts, and both reduces release of RANKL and increases that of OPG, thereby reducing osteoclastogenesis and osteoclast activation.[5,8] It is thus notable that following SCI, *ex vivo* cultures of osteoblasts, demonstrated reduced expression of several genes for Wnt signaling, specifically Wnt3a, Tcf7, Fzd5, and LRP5. These changes in expression of Wnt-signaling genes were accompanied by reduced expression of the Wnt-responsive genes ENC1[16] and Runx2.[5,8] Such changes would be anticipated to exacerbate the known effects of unloading of bone to upregulate the expression by osteoblasts and osteocytes of sclerostin and DKK1, two Wnt inhibitors.[3] These findings are consistent with those reported after a microarray analysis of osteoblasts.[17] Thus, collectively, these alterations raise the possibility that following SCI, there is a reduction in Wnt signaling in osteoblasts in sublesional bone after SCI.

Effect of nandrolone on bone loss after SCI

Our study demonstrated for the first time that nandrolone reduced bone loss after SCI. Additional evidence

of the relevance of androgens to bone loss after skeletal unloading is provided by findings that deletion of the androgen receptor increased bone loss and levels of markers of bone resorption in a mouse model of hind limb suspension[18] and that nandrolone reduced bone loss after nerve transection or microgravity.[11,12] This finding is particularly important in the setting of SCI because of the prevalence of hypogonadism in men with SCI, particularly early after injury.[1,2] It should be noted that in our study nandrolone did not restore normal bone mass after SCI. Whether this reflects an inability to completely prevent bone loss after SCI as opposed to lack of effectiveness in reversing bone loss that occurred during the first 28 days after injury cannot be determined from our findings. An interesting question is whether greater protection against bone loss would result if administration of nandrolone was begun at the time of SCI.

Molecular mechanisms of nandrolone action after SCI

Cell culture studies suggest that androgens increase canonical Wnt signaling in cells of the osteoblast lineage and, thereby, promote osteoblastic differentiation.[13] Several findings from this study indicated that nandrolone increased expression of Wnt signaling after SCI. In *ex vivo* osteoblast cultures, nandrolone increased expression of the Wnt-signaling genes Wnt3a, Fzd5, LRP5, and Tcf7. Nandrolone also increased the expression of three Wnt/β-catenin-responsive genes (Runx2, OPG, and ENC1), all of which were downregulated by SCI. Runx2 is an early differentiation factor in osteoblastogenesis of mesenchymal progenitor cells,[3] and ENC1 has also been proposed to promote such differentiation (John Whitehead, personal communication). These findings indicate that nandrolone increased Wnt signaling in *ex vivo*-cultured osteoblasts and suggest that nandrolone increases Wnt signaling in unloaded bone after SCI. We expect that gene expression changes in osteocytes may closely resemble those in *ex vivo*-cultured osteoblasts, and this will be an interesting area for future study.

In *ex vivo* cultures of osteoclasts at 56 days after SCI, nandrolone reduced expression of TRAP and calcitonin receptor, suggesting inhibitory effects on osteoclastogenesis and/or activity. Such effects of androgens on osteoclastogenesis and/or activity may be, at least in part, result from androgen-mediated activation of Wnt signaling and subsequent increase in OPG expression relative to that of RANKL. This notion is supported by the evidence that testosterone increased OPG expression in mouse 3T3-E1 cells.[19]

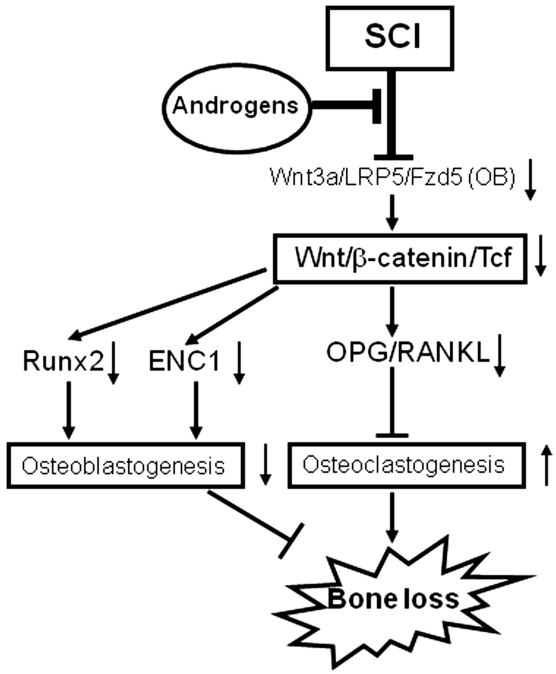


Figure 5 A scheme illustrating potential mechanisms by which nandrolone reduces bone loss after SCI. Small arrows indicate the direction of change after SCI. The findings of our study suggest that nandrolone increases levels of Wnt3a, LRP5, and Fzd5, and activates Wnt-signaling pathway, resulting in an increase in OPG/RANKL ratio, thereby inhibiting osteoclast differentiation. These effects of nandrolone are accompanied by increases in expression of RunX2 and ENC1, which may act by promoting osteoblast differentiation. These effects lead to a protection against bone loss after SCI.

Conclusions and clinical significance

The major finding of this study is that nandrolone reduced bone loss in a rat model of hind limb paralysis caused by a complete SCI. This finding is of potential clinical relevance because bone loss in individuals with motor complete SCI is rapid, severe, and refractory to bisphosphonates.[2] The possibility that a therapy with androgens might slow such loss is thus exciting. Our findings suggest a potential mechanism by which androgens reduce bone loss after SCI through the activation of Wnt signaling, leading to increased OPG expression and reduced osteoclastogenesis and osteoclast activity, as well as, potentially, increased osteoblastogenesis and osteoblast activity (Fig. 5). 4

Acknowledgements

This work was supported by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Rehabilitation Research and Development Service Grants B3347K, B4616R, and B4162C, the Department of Defense

Grant SC090504, and NIH Grants (AG23176, DK 70525, and DK 80459).

References

1 Qin W, Bauman WA, Cardozo C. Bone and muscle loss after spinal cord injury: organ interactions. Ann N Y Acad Sci 2010;1211(1): 66–84.
2 Qin W, Bauman WA, Cardozo CP. Evolving concepts in neurogenic osteoporosis. Curr Osteoporos Rep 2010;8(4):212–8.
3 Zaidi M. Skeletal remodeling in health and disease. Nat Med 2007; 13(7):791–801.
4 Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. Nat Med 2011;17(10):1235–41.
5 Kubota T, Michigami T, Ozono K. Wnt signaling in bone metabolism. J Bone Miner Metab 2009;27(3):265–71.
6 Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, *et al.* Mechanical stimulation of bone *in vivo* reduces osteocyte expression of Sost/sclerostin. J Biol Chem 2008;283(9): 5866–75.
7 Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. J Biol Chem 2005; 280(39):33132–40.
8 Glass DA, 2nd, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, *et al.* Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell 2005;8(5):751–64.
9 Erben RG, Eberle J, Stahr K, Goldberg M. Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study. J Bone Miner Res 2000;15(6):1085–98.
10 Smith MR, Fallon MA, Goode MJ. Cross-sectional study of bone turnover during bicalutamide monotherapy for prostate cancer. Urology 2003;61(1):127–31.
11 Wimalawansa SM, Chapa MT, Wei JN, Westlund KN, Quast MJ, Wimalawansa SJ. Reversal of weightlessness-induced musculoskeletal losses with androgens: quantification by MRI. J Appl Physiol 1999;86(6):1841–6.
12 Cardozo CP, Qin W, Peng Y, Liu X, Wu Y, Pan J, *et al.* Nandrolone slows hindlimb bone loss in a rat model of bone loss due to denervation. Ann N Y Acad Sci 2010;1192:303–6.
13 Liu XH, Kirschenbaum A, Yao S, Levine AC. Androgens promote preosteoblast differentiation via activation of the canonical Wnt signaling pathway. Ann N Y Acad Sci 2007;1116:423–31.
14 National Spinal Cord Injury Statistical Center. Spinal cord injury: facts and figures at a glance. J Spinal Cord Med 2011;34(6):620–1.
15 Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, *et al.* TSH is a negative regulator of skeletal remodeling. Cell 2003;115(2):151–62.
16 Fujita M, Furukawa Y, Tsunoda T, Tanaka T, Ogawa M, Nakamura Y. Up-regulation of the ectodermal-neural cortex 1 (ENC1) gene, a downstream target of the beta-catenin/T-cell factor complex, in colorectal carcinomas. Cancer Res 2001; 61(21):7722–6.
17 Jiang SD, Yan J, Jiang LS, Dai LY. Down-regulation of the Wnt, estrogen receptor, insulin-like growth factor-I, and bone morphogenetic protein pathways in osteoblasts from rats with chronic spinal cord injury. Joint Bone Spine 2011;78(5):488–92.
18 Saita Y, Nakamura T, Mizoguchi F, Nakashima K, Hemmi H, Hayata T, *et al.* Combinatory effects of androgen receptor deficiency and hind limb unloading on bone. Horm Metab Res 2009;41(11):822–8.
19 Chen Q, Kaji H, Kanatani M, Sugimoto T, Chihara K. Testosterone increases osteoprotegerin mRNA expression in mouse osteoblast cells. Horm Metab Res 2004;36(10):674–8.

Authors Queries

Journal: **The Journal of Spinal Cord Medicine**
Paper: **JSCM-D-12-00035**
Article title: **Anabolic steroids reduce spinal cord injury-related bone loss in rats associated with increased Wnt signaling**

Dear Author
During the preparation of your manuscript for publication, the questions listed below have arisen.
Please attend to these matters and return this form with your proof. Many thanks for your assistance

Query Reference	Query	Remarks
1	Please confirm the change of “expressed” to “voided” in the sentence “Urine was … wound infection”.	
2	Please provide the expansion for α-MEM.	
3	Please confirm the insertion of part labels (A and B) in Figure 1 caption.	
4	We have changed citation of Scheme 1 to Figure 5. Please confirm.