Orthopedic & Muscular System: Current Research

Xu et al., Orthop Muscular Syst 2016, 5:3
http://dx.doi.org/10.4172/2161-0533.1000220

Research Article Open Access

# Hydrogel-Based Local Release of Salubrinal Stimulates Healing of Mouse Tibia Fracture

**Wenxiao Xu**[1, 2], **Andy Chen**[3], **Yijia Zhang**[1], **Jiliang Li**[4], **Chien-Chi Lin**[1], **Jing-long Yan**[2] **and Hiroki Yokota**[1*]

[1]*Department of Biomedical Engineering, Indiana University Purdue University Indianapolis, Indianapolis, USA*

[2]*Department of Orthopedics, Second Affiliated Hospital of Harbin Medical University, Harbin, China*

[3]*Weldon School of Biomedical Engineering, Purdue University, West Lafayette, USA*

[4]*Department of Biology, Indiana University Purdue University Indianapolis, Indianapolis, USA*

[*]**Corresponding author**: Hiroki Yokota, Department of Biomedical Engineering, Indiana University Purdue University Indianapolis, 723 West Michigan Street, SL220 Indianapolis, IN 46202, USA, Tel: 317-278-5177; Fax: 317-278-2455; E-mail: hyokota@iupui.edu

**Received date**: June 10, 2016; **Accepted date**: July 01, 2016; **Published date**: July 08, 2016



## Abstract

**Background**: Salubrinal is a small synthetic agent that presents beneficial effects on skeletal diseases and tumor progression. It is reported to stimulate bone formation and suppress bone resorption. In this study, we examined whether salubrinal administration can stimulate the healing of bone fracture using a mouse model of closed tibia fracture.

**Materials and Methods:** We administered salubrinal to mice in two different routes: one-time hydrogel injection with salubrinal-loaded Poly Lactic-Co-Glycolic Acid (PLGA) microparticles; and daily subcutaneous injection for 4 weeks. A subcutaneous injection of Bone Morphogenetic Protein 2 (BMP2) was used as a positive control. The measurement of Bone Mineral Density (BMD)/Bone Mineral Content (BMC), as well as micro-CT imaging and mechanical testing were utilized to evaluate the healing of the experimental fracture.

**Results**: It was shown that 4 weeks after the induction of tibia fracture no groups, including the BMP2 control group, elevated BMD or BMC. Hydrogel-based injection of salubrinal showed a higher stiffness than that of the vehicle control, as well as significant elevation of ultimate force. Although daily subcutaneous injection of BMP2 increased stiffness and ultimate force, daily injection of salubrinal did not show significant improvement of mechanical properties. Of note, the total salubrinal dose in the hydrogel group was approximately 18% of that in the subcutaneous group.

**Discussion**: Improvement in mechanical properties by a hydrogel-based administration of salubrinal and not by a daily subcutaneous injection indicates dependence of salubrinal's efficacy on its administration procedure. Salubrinal is capable of suppressing tumor growth, a clear advantage over a growth factor such as BMP2. For a future clinical trial, administration frequency and optimal dosage may need to be further analyzed.

**Keywords:** Bone fracture; Tibia; Salubrinal; Hydrogel; Mechanical test

## Introduction

To stimulate the healing of bone fracture, various pharmacological therapies have been investigated. The administration of Bone Morphogenetic Proteins (BMPs) has been shown in randomized controlled trials to be efficient in tibial fracture healing [1,2], and Vascular Endothelial Growth Factor (VEGF) is also reported to enhance bone healing [3]. Systemic therapy with agents such as Parathyroid Hormone (PTH), Growth Hormone (GH), and the HMG-CoA reductase inhibitors are under investigation. Through Wnt signaling, sclerostin antibody was shown to increase bone formation, bone mass, and bone strength [4]. Molecules such as prostaglandin E receptor agonists and the thrombin-related peptide, TP508, have presented promise in animal models of fracture repair [5,6], and a possibility of gene therapy such as BMP2-transfected bone marrow was demonstrated [7]. However, difficult healing problems, such as delayed union, nonunion, growth abnormalities, and infection, require more effort to improve existing therapies in clinic.

Salubrinal is a synthetic compound (C21H17Cl3N4OS; 480 Da) which is known to reduce various cellular stresses including stress to the endoplasmic reticulum [8,9]. It inhibits serine/threonine protein phosphatase 1 alpha (PP1), followed by the elevation of phosphorylated eukaryotic translation initiation factor 2 alpha (eIF2α) [10]. Salubrinal is reported to enhance bone formation by stimulating Activating Transcription Factor 4 (ATF4), one of the transcription factors for bone formation, via eIF2α-mediated signaling and stimulating development of bone-forming osteoblasts [11]. It also suppresses nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), a master transcription factor for osteoclastogenesis, and inhibits development of bone-resorbing osteoclasts [12,13]. It reduces inflammation and degradation of cartilage tissues [14,15], and it is capable of attenuating proliferation and migration of breast cancer cells [16].

While salubrinal can add calcified mass to osteoporotic bone, its effect on the healing of bone fracture has not been tested. The current

study investigated efficacy of salubrinal in the healing of tibia fracture using a mouse closed fracture model [17]. We have previously examined the effects of salubrinal using a rat surgical wound model [18]. However, the healing of surgical holes does not represent an orchestrated process of fracture healing by many types of cells, including mesenchymal stem cells and hematopoietic stem cells, as well as osteoblasts and osteoclasts [19,20].

A specific question, addressed herein, was: does salubrinal's delivery method change its efficacy in facture healing? Two different delivery methods were employed in this study: single *in situ* injection of Poly(Ethylene Glycol) (PEG) hydrogel with salubrinal-loaded Poly(Lactic-Co-Glycolic Acid) (PLGA) microparticles; and daily subcutaneous injection. Hydrogels are highly hydrated networks of crosslinked polymer chains [21]. PEG-based hydrogels provide high degrees of tunability in matrix modification [22], and might have the benefits to emulate native matrix mechanics as well as to deliver therapeutic agents. In this study, salubrinal-loaded PLGA microparticles were mixed with PEG macromers capable of *in situ* gelling, and the composite was injected and cured *in situ*.

## Methods and Materials

### Animal preparation

Seventy-two C57BL/6 female mice (14 weeks, body weight ~20 g; Harlan Sprague–Dawley Inc., Indianapolis, IN, USA) were used in the study. All procedures performed in this study were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Five mice were housed together in a cage. Animals were fed with standard laboratory chow and water ad libitum, and they were allowed to acclimate for 1 week before experimentation. Mice were divided into 6 groups, in which group 1 was treated as normal control (no induction of tibia fracture). Groups 2 and 3 were used for testing hydrogel-based administration of vehicle and salubrinal, while groups 4-6 for examining subcutaneous injection of vehicle, BMP2 and salubrinal.

### Surgical procedure for induction of the closed tibia fracture

Prior to the induction of closed fracture in the right tibia, mice were anesthetized using 1.5% isoflurane. To mice in groups 2-5, a stainless steel wire (30-gauge needle) was inserted into the intramedullary cavity of the right tibia through its proximal end (Figure 1C and 1D). The wire extending beyond the tibia condyles was cut, and the patella was properly repositioned. A closed diaphyseal fracture was then induced in the right distal tibia using a custom made 3-point bending device with a consistent force (Figure 1A) [23]. Radiographic images were taken (Faxitron, Tucson, AZ, USA) on day 0, as well as weeks 1, 2, 3 and 4 after fracture induction (Figure 1C and 1D).

### Fabrication of Salubrinal-loaded PLGA microparticles

Salubrinal loaded PLGA (50:50, MW: 30-60 kDa, Sigma) microparticles were prepared using oil-in-water emulsion method [24]. First, 100 mg/ml of PLGA was dissolved in Dichloromethane (DCM). Next, 2 mg of salubrinal was added to 1 ml of PLGA/DCM solution, followed by vortexing for 1 minute. The primary emulsion was added to 2 ml of 1% Poly(Vinyl Alcohol) (PVA) solution and vortexed for 3 minutes. The secondary emulsion was poured into 20ml of aqueous solution containing 0.5% PVA and 450 mM sodium chloride. The emulsion was stirred for 4 hours at 700 rpm to allow the evaporation of DCM, and the hardened microparticles were collected by centrifugation (2000 rpm, 5 min) and washed three times with ddH2O. The drug-loaded PLGA microparticles were then freeze dried and stored at -20°C until use.

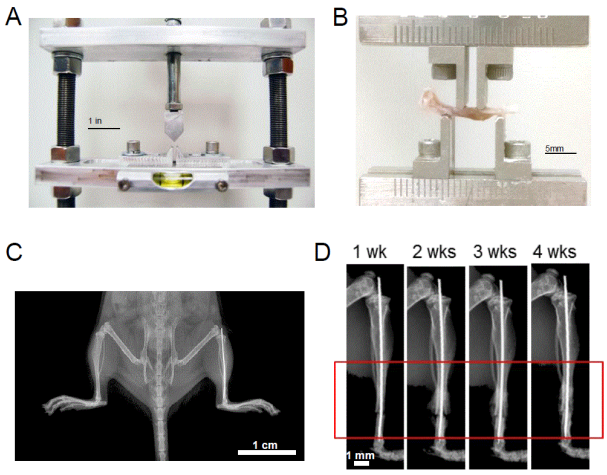


**Figure 1**: Tibia fracture study. (A) Custom-made 3-point bending device for inducing closed tibia fracture. (B) Four-point bending setup for mechanical testing. (C and D) X-ray images of the fractured tibiae. Of note, a stainless steel rod was inserted in the medullary cavity of the tibia.

### *In situ* rheometry of PEG hydrogelation with and without drug-loaded PLGA microparticles

*In situ* cured PEG hydrogels were prepared by reacting four-arm PEG-acrylate (PEG4A, 20 kDa, synthesized using published protocol) [22] and four-arm PEG-thiol (PEG4SH, 10 kDa, purchased from JenKem Technology USA) (Figure 2A) through Michael-type addition reaction [25] (Figure 2B). Briefly, stock solutions of PEG4A and PEG4SH (both at 20 wt%) were mixed at equal volume and pipetted onto the platform of a digital rheometer (CVO 100, Malvern). Eight-mm parallel plate geometry was used, and the gelation was monitored using single frequency rheometry (1 Hz) operated at 5% strain. In one group, PLGA microparticles (10 wt%) were mixed with the PEG solutions prior to *in situ* rheometry measurement.

### Administration of salubrinal and BMP2

Each group contained 12 mice, and group 1 was treated as a normal control. Mice in group 2 (hydrogel control) were given hydrogel without any agent on day 1, while mice in group 3 (hydrogel salubrinal) received a single administration of salubrinal loaded hydrogel (100μg of salubrinal in 50 μl of gel; equivalent to a single injection at a dose of 5 mg/kg). Mice in group 4 (fracture control) received daily subcutaneous injections of the vehicle (50 μl) to the fracture site, while mice in group 5 (subcutaneous BMP2) were given daily injections of BMP2 (10 μg/kg in 50 μl of vehicle) as a positive control. Lastly, mice in group 6 (subcutaneous salubrinal) received daily injections of salubrinal (1 mg/kg in 50 μl of vehicle).

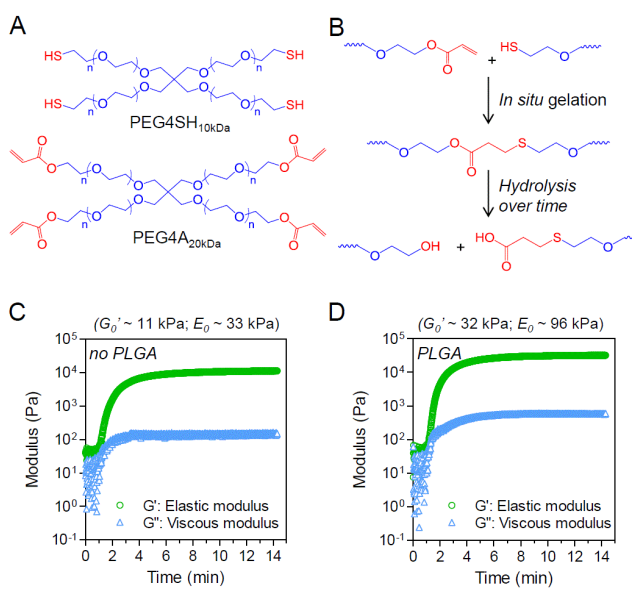


**Figure 2**: Hydrogel based delivery. (A) Chemical structure of two PEG derivatives (PEG4SH and PEG4A). (B) Chemical reactions for in situ gelation and overtime hydrolysis. (C) Changes in moduli during gelation without PLGA particles. (D) Changes in moduli during gelation with salubrinal-loaded PLGA particles.

### Measurements of bone mineral density (BMD) and bone mineral content (BMC)

Mice were sacrificed 4 weeks after the fracture induction, and tibiae were harvested. Isolated tibiae were cleaned of soft tissues and stored at -20°C in gauze that was moisturized with PBS. The BMD and BMC of the entire tibia and callus region at the fracture site were determined using a PIXImus densitometer with a threshold value of 1800 (software version 1.4; GE Medical System Lunar, Madison, WI, USA) [26]. The callus region was defined as a fixed rectangle region of interest (20 pixels × 20 pixels) that covered the callus center.

### Fracture score

The fracture scores were defined from 0 to 3: "0" for no fracture, "1" for minor fracture, "2" for moderate fracture, and "3" for major fracture. X-ray images of the fractured tibia at weeks 0-4 were randomly labeled, and eight independent scorers participated in the evaluation. All scores were collected and averaged for each sample and time point.

### Micro CT imaging

Micro-computed tomography was performed using Skyscan 1172 (Bruker-MicroCT, Kontich Belgium) [27]. The harvested tibiae were wrapped in parafilm to maintain hydration and placed in a plastic tube and oriented vertically. Scans were performed at pixel size 8.99 μm. Using manufacturer-provided software, the images were reconstructed (nRecon v1.6.9.18), cross sections were obtained (Dataviewer, v1.5.0), and 3D models were generated (CT Analyser, v1.11.4.2) and visualized (CTvol, v2.2.3.0).

### Mechanical testing of the tibia

Tibiae were tested to failure by four-point bending using a voltage-regulated mechanical loading device (ElectroForce 3100, Bose, Inc.), with a loading span of 2.3 mm and a support span of 7 mm (Figure 1B) [28]. The load was applied to the medial tibia such that the right span was located just inside of the tibia-fibula junction. After preloading to 0.5 N, the bone was twice loaded with a sinusoidal regimen of 0.5 Hz, 1 Hz, and 2 Hz at amplitude 1 N. The bone was then loaded monotonically at 0.005 mm/s until failure. Load and displacement were recorded and used to calculate stiffness and ultimate force.

### Statistical analysis

The data were expressed as mean ± SEM. Statistical significance among groups was examined using one-way Analysis of Variance (ANOVA), and a post hoc test was conducted using Fisher's Protected Least Significant Difference (PLSD) for the pairwise comparisons. Statistical significance was assumed for $p < 0.05$.

## Results

### *In situ* rheometry of gelation with and without drug-loaded PLGA microparticles

We first monitored the gelation process through *in situ* rheometry either in the absence (Figure 2C) or presence (Figure 2D) or PLGA microparticles. As expected, the two PEG macromers, PEG4A and PEG4SH, reacted to form a crosslinked network, demonstrated by rapid crossover of hydrogel elastic modulus (G') and viscous modulus (G") with a gel point of less than 1 minute regardless of the presence of PLGA microparticles. The gelation reached near completion within a few minutes of mixing the two macromer components. This timing is ideal as it allowed sufficient time to mix the essential components (i.e., PEG4A, PEG4SH, and PLGA microparticles) while permitting rapid gelation locally at the fracture site. It was worth noting that the addition of PLGA microparticles increased the Young's modulus (E0) of the resulting hydrogels from ~33 kPa to ~96 kPa (Figure 2C and 2D).

### No significant change in BMD/BMC by hydrogel-based salubrinal administration

Regarding the hydrogel-based administration of salubrinal, micro CT images of the representative tibia samples after harvest in week 4 were captured (Figure 3A-3C). Although X-ray images in week 4 indicated a complete bridge of the fracture site with calcified tissue, the sagittal sections of micro CT images revealed discontinuous cortical bone at the fracture site. The BMD and BMC measurement of the entire tibia in week 4 did not show any significant changes in the hydrogel control (group 2) and salubrinal (group 3) samples. The same measurement in the restricted callus region in week 4 presented a tendency of increase in the salubrinal treated group, but the difference was not statistically significant ($p = 0.2$ for BMD, and $p = 0.09$ for BMC) (Figure 3D and 3E).

The fracture score, which indicated the degree of discontinuity in the cortical bone at the fracture site, decreased in groups 2 and 3 (Figure 4A). No statistical difference was observed during the 4-week healing period between the two groups except for week 2.

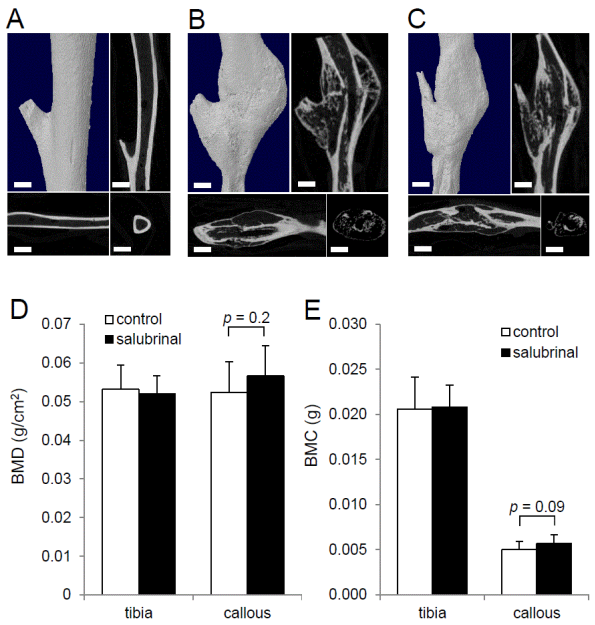


**Figure 3**: Micro CT images, and BMD and BMC for groups 1-3. (A) Micro CT images of the normal control sample (group 1). Scale bar indicates 1 mm. (B) Micro CT images of the hydrogel control sample (group 2). (C) Micro CT images of the hydrogel salubrinal sample (group 3). (D) BMD for the whole tibia and callous near the fracture site for groups 2 and 3. (E) BMC for the whole tibia and callous near the fracture site for groups 2 and 3.

## Elevated ultimate force by hydrogel-based salubrinal administration

The force-displacement relationship for 3 groups showed a distinctively different profile in response to a sinusoidal load (0.5 N, peak-to-peak) at 0.5, 1, and 2 Hz (Figure 4B–4G). The ascending and descending loads exhibited the same pattern, in which the intact control (group 1) was the stiffest and the hydrogel control (group 3) was the softest with the hydrogel salubrinal (group 2) in between. The ultimate force was 11.64 ± 5.48 N (hydrogel control, n = 12) and 16.99 ± 5.41 N (hydrogel salubrinal, n = 12) with p = 0.03, while stiffness was 81.55 ± 23.58 N/mm (hydrogel control, n = 12) and 106.73 ± 32.81 N/mm (hydrogel salubrinal, n = 12) with p = 0.05 (Figure 5).

## Differential effects of hydrogel-based administration and subcutaneous salubrinal injection

Regarding daily subcutaneous injection of vehicle (group 4), BMP2 (group 5), and salubrinal (group 6), the fracture score was determined for weeks 0 to 4 using longitudinal X-ray images (Figure 6A).

Images collected by PIXImus densitometer were used for determining BMD and BMC (Figure 6B and 6C). The image analysis revealed that no statistical difference was detected among three groups for the fracture score, BMD, and BMC. Mechanical test using 4-point bending revealed that ultimate force and stiffness were significantly elevated in group 5 (subcutaneous BMP2), but no statistical difference was detected between group 4 (fracture control) and group 6 (subcutaneous salubrinal) (Figure 6D and 6E).

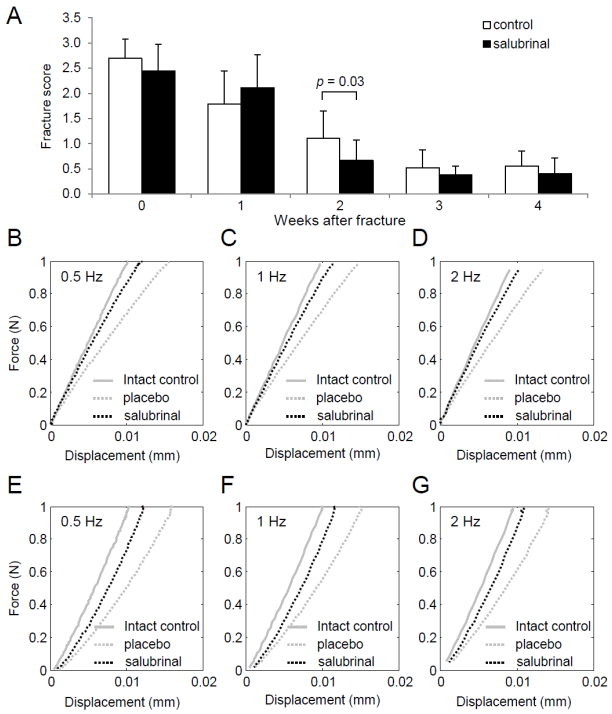


**Figure 4**: Fracture score and force-displacement relationship for groups 1-3. (A) Fracture score in weeks 0-4 for the hydrogel control and hydrogel salubrinal groups. Of note, fracture score = "0" (no obvious fracture), "1" (minor fracture), "2" (moderate fracture), and "3" (severe fracture). (B-D) Force-displacement relationship for groups 1-3 in response to an increasing sinusoidal force (0.5 N, peak-to-peak) at 0.5, 1, and 2 Hz, respectively. (E-G) Force-displacement relationship for groups 1-3 in response to a decreasing sinusoidal force (0.5 N, peak-to-peak) at 0.5, 1, and 2 Hz, respectively.

## Discussion

This study demonstrates that hydrogel-based administration of salubrinal elevates the ultimate force in the four-point bending test and improves mechanical strength of the fractured tibia. The PEG-based hydrogels used in this study were prepared using multi-arm PEG macromers with mutually reactive acrylate and thiol moieties.

*In situ* gelation was achieved through a Michael-type addition reaction, and the resulting thiolether ester bonds could be degraded hydrolytically and/or enzymatically by esterases. We fabricated degradable PLGA microparticles for loading and subsequently releasing salubrinal *in vivo*. After injecting the precursor solution in the fracture site, hydrogel hardened and effectively entrapped the drug-loaded microparticles. Instead of delivering as daily systemic injections, the hydrogel served as void filling matrices, whereas the PLGA microparticles carried salubrinal and released it locally and gradually. Ideally, the PEG hydrogels and the PLGA microparticles would degrade slowly as the new bone formed and regenerated.

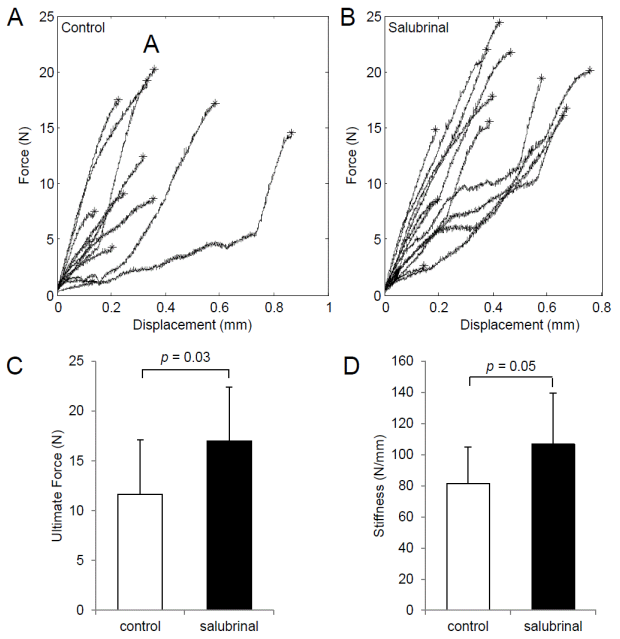


**Figure 5**: Mechanical strength 4 weeks after fracture induction in groups 1-3. (A) Force-displacement relationship for the hydrogel control group in response to a linearly increasing displacement. The cross indicates the site of ultimate force. (B) Force-displacement relationship for the hydrogel salubrinal group. (C) Ultimate force of the hydrogel control and hydrogel salubrinal groups. (D) Stiffness of the hydrogel control and hydrogel salubrinal groups.

In a local delivery with a single injection, salubrinal was loaded in PLGA microparticles and given one day after the induction of tibia fracture at a dose of 5 mg/kg. When salubrinal was applied as a daily subcutaneous injection at a dose of 1 mg/kg, no significant improvement of mechanical parameters such as the ultimate force and stiffness was observed. Since the subcutaneous injection was conducted 28 times during the 4-week healing period, the total dose became 28 mg/kg. Collectively, efficacy of salubrinal in fracture healing significantly depends on its delivery method. A local delivery with a dose of 5 mg/kg was found to be more effective than a systemic delivery with a total dose of 28 mg/kg.

Agents for the healing of bone fracture are primarily screened and selected based on their efficacy in bone formation and bone remodeling [29]. Salubrinal is reported to suppress Lipopolysaccharide (LPS)-Stimulated inflammatory responses in macrophages [14], reduce orofacial inflammatory pain [30], and alleviates colitis through suppression of pro-inflammatory cytokines [31]. Furthermore, it attenuates inflammatory cytokines such as IL1α, Cox2, IL2, TNF, and IL13 in macrophages, T lymphocytes, and mast cells [14]. The mechanism of salubrinal's anti-inflammatory action and its link to eIF2α regulation are not satisfactorily understood [32]. Besides eIF2α-mediated signaling, salubrinal is reported to downregulate NFαB signaling in an eIF2α-independent fashion [15]. Although inflammatory responses are a critical phase in fracture healing, further analysis is necessary to examine whether salubrinal's potential suppression of inflammatory responses may accelerate the overall healing of fractured bone.

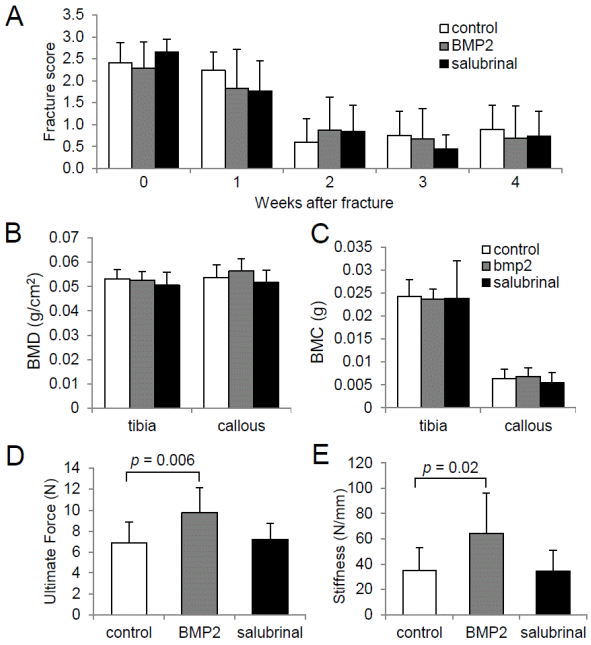


**Figure 6**: Administration of BMP2 and salubrinal as subcutaneous injection near the fracture site in groups 4-6. (A) Fracture score in weeks 0-4 for the subcutaneous vehicle, BMP2, and salubrinal groups. (B & C) BMD and BMC of the fractured tibia, respectively. (D) Ultimate force of the subcutaneous control, BMP2, and salubrinal samples. (E) Stiffness of the subcutaneous control, BMP2, and salubrinal samples.

While BMP2 mainly enhances bone formation without directly affecting bone resorption, salubrinal not only stimulates osteoblastogenesis but also inhibits osteoclastogenesis in the reparative and remodeling phases of fracture healing. Furthermore, salubrinal's action is mediated using its unique signaling pathway. We have previously shown that salubrinal activates development of osteoblasts by upregulating ATF4, followed by ATF4-mediated elevation of osteocalcin. Upregulation of ATF4 is primarily driven by the elevated eIF2α phosphorylation [10], while BMP2 activates many other signaling pathways including Smad, Mitogen-Activated Protein Kinase (MAPK), Wnt, Hedgehog, Notch, and Fibroblast Growth Factor (FGF) [33]. Regarding bone resorption, our previous studies demonstrate that salubrinal suppresses the proliferation and maturation of osteoclasts by downregulating AP-1 proteins such as c-Fos and JunB, as well as NFATc1 [12].

The mechanistic differences in the actions of BMP2 and salubrinal may result in their differential effects on malignant tumor. BMP2 has a risk of inducing cancer [34], but salubrinal is reported to present an inhibitory role in growth and migration of breast cancer cells [16]. Bisphosphonates are anti-resorptive agents which have been frequently administered for treatment of osteoporosis. In bone-fractured animals treated with bisphosphonates, an increase in callus size and bone strength is reported [35]. A comparative study between bisphosphonates and salubrinal might help understand significance of

anti-resorptive actions in the fracture healing. There are several factors that might contribute to further elevating salubrinal's efficacy. First, the most effective release rate of salubrinal might be determined by adjusting its loading condition to PLGA. Second, the size of PEG molecules (currently 10 kDa and 20 kDa) might be altered to control PLGA retention in a hydrogel and salubrinal's release rate. Third, salubrinal's efficacy may affect each of the three healing phases differently.

In this study, we focused on mechanical testing for evaluating salubrinal's delivery methods since mechanical properties such as stiffness and ultimate force are practical measures of efficacy in the healing of bone fracture. Unlike BMP2, salubrinal is capable of suppressing tumor growth. Although an increase in BMD can be viewed as a positive outcome of anabolic responses, it could also be interpreted as evidence for ectopic bone formation and delayed bone remodeling. In summary, we demonstrated that single hydrogel-based administration of salubrinal increased mechanical strength of the fractured tibia. Further analysis regarding administration frequency and optimal dosage may warrant a future clinical trial to the common closed tibial shaft fracture.

## Authorship

Xu W, Zhang Y, Chi Lin C and Yokota H designed this study. Xu W, Zhang Y, Chi Lin C and Chen A contributed to the data collection. W, Zhang Y, Chi Lin C and Chen A and Yokota H and Yokota H performed the data analysis. Xu W, Zhang Y, Chi Lin C, long Yan J, Li J and Yokota H interpreted the data. Xu W, Chen A, Zhang Y, Chi Lin C and Yokota H wrote the manuscript. All authors contributed to the critical revision.

## Disclosure

There was no funding from the National Institutes of Health (NIH), Welcome Trust, or the Howard Hughes Medical Institute (HHMI) for this work. No conflicts of interest were declared by any of the authors. The Department of Defense provided funding for this study (DOD W81XWH-11-1-0716).

## Acknowledgements

The authors appreciate Kazumasa Minami, Yu Sun, Shinya Takigawa, Shengzhi Liu, Allen Zhang, and Allen Zhou for technical support.

## References

1. Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, et al. (2002) Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg Am 84: 2123-2134.
2. Schmidmaier G, Wildemann B, Ostapowicz D, Kandziora F, Stange R, et al. (2004) Long-term effects of local growth factor (IGF-I and TGF-beta 1) treatment on fracture healing. A safety study for using growth factors. J Orthop Res 22: 514-519.
3. Eckardt H, Ding M, Lind M, Hansen ES, Christensen KS, et al. (2005) Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model. J Bone Joint Surg Br 87:1434-1438.
4. Li X, Ominsky MS, Warmington KS, Morony S, Gong J, et al. (2009) Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. J Bone Miner Res 24: 578-588.
5. Paralkar VM, Borovecki F, Ke HZ, Cameron KO, Lefker B, et al. (2003) An EP2 receptor-selective prostaglandin E2 agonist induces bone healing. Proc Natl Acad Sci USA 100: 6736-6740.
6. Sheller MR, Crowther RS, Kinney JH, Yang J, Di Jorio S, et al. (2004) Repair of rabbit segmental defects with the thrombin peptide, TP508. J Orthop Res 22: 1094-1099.
7. Lieberman JR, Daluiski A, Stevenson S, Wu L, McAllister P, et al. (1999) The effect of regional gene therapy with bone morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. J Bone Joint Surg Am 81: 905-917.
8. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, et al. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science 307: 935-939.
9. Ron D (2002) Translational control in the endoplasmic reticulum stress response. J Clin Invest 110: 1383-1388.
10. Wek RC, Jiang HY, Anthony TG (2006) Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34: 7-11.
11. Yokota H, Hamamura K, Chen A, Dodge TR, Tanjung N, et al. (2013) Effects of salubrinal on development of osteoclasts and osteoblasts from bone marrow-derived cells. BMC Musculoskelet Disord 14: 197.
12. Hamamura K, Tanjung N, Yokota H (2013) Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha. J Bone Miner Metab 31: 618-628.
13. Hamamura K, Chen A, Tanjung N, Takigawa S, Sudo A, et al. (2015) *In vitro* and in silico analysis of an inhibitory mechanism of osteoclastogenesis by salubrinal and guanabenz. Cell Signal 27: 353-362.
14. Hamamura K, Nishimura A, Chen A, Takigawa S, Sudo A, et al. (2015) Salubrinal acts as a Dusp2 inhibitor and suppresses inflammation in anti-collagen antibody-induced arthritis. Cell Signal 27: 828-835.
15. Hamamura K, Nishimura A, Iino T, Takigawa S, Sudo A, et al. (2015) Chondroprotective effects of Salubrinal in a mouse model of osteoarthritis. Bone Joint Res 4: 84-92.
16. Hamamura K, Minami K, Tanjung N, Wan Q, Koizumi M, et al. (2014) Attenuation of malignant phenotypes of breast cancer cells through eIF2α-mediated downregulation of Rac1 signaling. Int J Oncol 44: 1980-1988.
17. Antonova E, Le TK, Burge R, Mershon J (2013) Tibia shaft fractures: costly burden of nonunions. BMC Musculoskelet Disord 14: 42.
18. Zhang P, Hamamura K, Jiang C, Zhao L, Yokota H (2012) Salubrinal promotes healing of surgical wounds in rat femurs. J Bone Miner Metab 30: 568-579.
19. Wehner T, Gruchenberg K, Bindl R, Recknagel S, Steiner M, et al. (2014) Temporal delimitation of the healing phases via monitoring of fracture callus stiffness in rats. J Orthop Res 32: 1589-1595.
20. Marsell R, Einhorn TA (2011) The biology of fracture healing. Injury 42: 551-555.
21. Gibbs DM, Black CR, Dawson JI, Oreffo RO (2016) A review of hydrogel use in fracture healing and bone regeneration. J Tissue Eng Regen Med 10: 187-198.
22. Lin CC (2015) Recent advances in crosslinking chemistry of biomimetic poly(ethylene glycol) hydrogels. RSC Adv 5: 39844-398583.
23. Manigrasso MB, O'Connor JP (2004) Characterization of a closed femur fracture model in mice. J Orthop Trauma 18: 687-695.
24. Ito F, Fujimori H, Honnami H, Kawakami H, Kanamura K, et al. (2010) Control of drug loading efficiency and drug release behavior in preparation of hydrophilic-drug-containing monodisperse PLGA microspheres. J Mater Sci Mater Med 21: 1563-1571.
25. Hao Y, Shih H, MuÅˆoz Z, Kemp A, Lin CC (2014) Visible light cured thiol-vinyl hydrogels with tunable degradation for 3D cell culture. Acta Biomater 10: 104-114.
26. Zhang P, Hamamura K, Turner CH, Yokota H (2010) Lengthening of mouse hindlimbs with joint loading. J Bone Miner Metab 28: 268-275.

27. Berman AG, Clauser CA, Wunderlin C, Hammond MA, Wallace JM (2015) Structural and Mechanical Improvements to Bone Are Strain Dependent with Axial Compression of the Tibia in Female C57BL/6 Mice. PLoS One 10: e0130504.
28. Hiltunen A, Vuorio E, Aro HT (1993) A standardized experimental fracture in the mouse tibia. J Orthop Res 11: 305-312.
29. Bukata SV (2011) Systemic administration of pharmacological agents and bone repair: what can we expect. Injury 42: 605-608.
30. Yang ES, Bae JY, Kim TH, Kim YS, Suk K, et al. (2014) Involvement of endoplasmic reticulum stress response in orofacial inflammatory pain. Exp Neurobiol 23: 372-380.
31. Okazaki T, Nishio A, Takeo M, Sakaguchi Y, Fukui T, et al. (2014) Inhibition of the dephosphorylation of eukaryotic initiation factor 2a ameliorates murine experimental colitis. Digestion 90: 167-178.
32. Hamamura K, Chen A, Uto Y, Yokota H (2015) Potential therapeutic applications of salubrinal for skeletal diseases and beyond. J Nat Sci 1: 151.
33. Wan M, Cao X (2005) BMP signaling in skeletal development. Biochem Biophys Res Commun 328: 651-657.
34. Carragee EJ, Chu G, Rohatgi R, Hurwitz EL, Weiner BK, et al. (2013) Cancer risk after use of recombinant bone morphogenetic protein-2 for spinal arthrodesis. J Bone Joint Surg Am 95: 1537-1545.
35. Li J, Mori S, Kaji Y, Mashiba T, Kawanishi J, et al. (1999) Effect of bisphosphonate (incadronate) on fracture healing of long bones in rats. J Bone Miner Res 14: 969-979.