Published in final edited form as:

J Orthop Res. 2016 December ; 34(12): 2106–2115. doi:10.1002/jor.23235.

Alcohol-Related Deficient Fracture Healing Is Associated With Activation of FoxO Transcription Factors in Mice

Philip M. Roper^{1,2}, Pegah Abbasnia¹, Aleksandra Vuchkovska², Roman M. Natoli³, and John J. Callaci^{1,2}

¹Department of Orthopaedic Surgery and Rehabilitation, Loyola University Medical Center, Maywood, Illinois ²Integrative Cellular Biology Program, Health Sciences Campus, Loyola University Chicago, Building 110, Room 4244, 2160 S First Ave, Maywood, Illinois ³University of Maryland Shock Trauma Center, Baltimore, Maryland

Abstract

The process of fracture healing is complex, and poor or incomplete healing remains a significant health problem. Proper fracture healing relies upon resident mesenchymal stem cell (MSC) differentiation into chondrocytes and osteoblasts, which are necessary for callus formation and ossification. Alcohol abuse is a leading contributor to poor fracture healing. Although the mechanism behind this action is unknown, excessive alcohol consumption is known to promote systemic oxidative stress. The family of FoxO transcription factors is activated by oxidative stress, and FoxO activation antagonizes Wnt signaling, which regulates mesenchymal stem cell differentiation. We hypothesize that alcohol exposure increases oxidative stress leading to deficient fracture repair by activating FoxO transcription factors within the fracture callus which disrupts chondrogenesis of mesenchymal stem cells. Our laboratory has developed an experimental model of delayed fracture union in mice using ethanol administration. We have found that ethanol administration significantly decreases external, cartilaginous callus formation, and hallmarks of endochondral ossification, and these changes are concomitant with increases in FoxO expression and markers of activation in fracture callus tissue of these mice. We were able to prevent these alcohol-induced effects with the administration of the antioxidant n-acetyl cysteine (NAC), suggesting that alcohol-induced oxidative stress produces the perturbed endochondral ossification and FoxO expression.

Keywords

alcohol; fracture; FoxO; Wnt; oxidative stress

Correspondence to: Philip M. Roper (T: 708-327-2416; F: 708-327-2813; proper@luc.edu).

Conflicts of interest: None.

AUTHORS' CONTRIBUTIONS

PMR is the corresponding and lead author for this manuscript, and has produced all of the data contained within. PA, AV, and RN provided laboratory, surgical, and data acquisition support, as well as manuscript proofreading. JJC is the principal investigator on the study. All authors have read and approved this manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

Bone fracture healing is the end result of a complex orchestration of molecular and cellular processes. Although most fractures heal normally, fracture nonunion is a significant clinical issue, with approximately 10% of long bone fractures, and up to 19% of tibia fractures, failing to heal normally.¹ Therefore, it is critical to gain a better understanding of the processes leading to abnormal fracture repair in order to find ways to bolster proper healing. A leading contributor to delayed and incomplete fracture union is alcohol abuse, which is also a well-known public health problem.^{2,3} Alcohol abusers have an increased risk of developing osteopenia, and have a fracture rate four times higher than that of non-abusers.^{3–6} Up to 40% of orthopedic trauma patients present with a positive blood alcohol content at the time of hospital admission, and alcohol consumption significantly raises the risk of healing complications, leading to nonunion and increased fracture healing times. Additionally, many trauma patients continue drinking after sustaining an injury.^{6–8} Basic science research using rodents has also shown that both acute and chronic alcohol administration have deleterious effects on bone health, as well as the ability to disrupt healing.^{4,5,9–12} Our laboratory has developed an experimental model of delayed fracture union in mice using a model of repeated episodic ethanol administration, as binge drinking, defined as reaching a blood alcohol concentration of 0.08 g/dl, has recently been shown to be more prevalent than chronic alcohol consumption, has highly significant deleterious health and social effects, and has a greater association with traumatic injury than chronic consumption.⁸

Long bone fracture injuries that are semi-rigidly stabilized tend to heal through both intramembranous and endochondral ossification, processes that rely on mesenchymal stem cells at the fracture to differentiate into chondrocytes and osteoblasts.¹³ At the initiation of endochondral ossification, chondrocytes are needed to produce cartilage and form the robust cartilaginous callus around the fracture site. This supplies the matrix that osteoblasts will eventually ossify, leading to a complete union. This whole process completely hinges on complex intracellular signaling cascades within resident MSCs to differentiate properly during the initiation of healing, and any perturbation of these pathways could have significant effects downstream.

Previous research into the effects of alcohol abuse on fracture healing has predominantly used models of osteotomies, focusing on late-stage mineralization or early changes in systemic and local inflammatory markers.^{5,9,14–18} While important, these studies fail to examine the effects of alcohol on a clinically-relevant model of fracture healing, leaving much to be elucidated about how alcohol affects early stages of healing, and the mechanisms behind these effects.

The metabolism of alcohol is known to contribute to increased systemic oxidative stress and reactive oxygen species (ROS) accumulation.¹⁹ Any increase in oxidative stress or shift in the redox state of the intracellular milieu can perturb key cellular mechanisms. One molecular responder to oxidative stress is the family of FoxO (forkhead box O) transcription factors, in which there are three predominant members, FoxO1, FoxO3, and FoxO4.^{20–22} Oxidative stress is shown to activate FoxOs, and once activated FoxOs regulate the expression of genes related to oxidative stress resistance and cell cycle inhibition.^{20,23} Increased oxidative stress, and the subsequent increase in FoxO activation, has been shown

to be detrimental to overall bone health.^{24,25} A reduction in FoxO expression lead to accelerated skin wound healing²⁶; however, the role of FoxO activation in fracture healing remains less well elucidated. To this point, our laboratory has devised a study to determine the effects alcohol has on specific callus components during fracture healing, and if alcohol has the ability to influence FoxO activity at any time during this process.

FoxO activation is especially intriguing to investigate during fracture healing because MSC differentiation towards chondrocytes and osteoblasts is controlled in large part by canonical Wnt signaling.^{27–30} Activation of canonical Wnt Signaling leads to the translocation of the transcription factor β -catenin into the nucleus where it can regulate specific target genes related to chondrocyte or osteoblast differentiation.^{27,28} Activated FoxOs bind β -catenin as a necessary cofactor in order to up regulate specific target genes related to oxidative stress resistance.³¹ Also, the ability of oxidative stress to activate FoxOs has been shown to lead to a decrease in Wnt/ β -catenin signaling, and preventing the accumulation of ROS has been shown to augment Wnt signaling and osteoblastogenesis in MSCs.^{24,31–35}

Our laboratory has shown that binge alcohol administration in rodents suppresses canonical Wnt signaling and the expression of Wnt target genes within the fracture callus, all while suppressing the formation of the external, cartilaginous callus,^{10,11,36,37} yet a discrete mechanism behind these deleterious effects has yet to be elucidated. Here, we hypothesize that episodic ethanol administration leads to deficient fracture repair by activating FoxO transcription factors within the fracture callus, which suppresses chondrogenesis and subsequent cartilaginous callus formation. We also hypothesize that administering an antioxidant, n-acetyl-cysteine (NAC), during healing to curb the accumulation of ROS would attenuate the deficiencies of fracture healing caused by alcohol.

METHODS

Alcohol Administration

Male C57Bl/6 mice 6–7 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) and housed in a facility approved by the Institutional Animal Care and Use Committee at Loyola University Medical Center. The mice were allowed to acclimate to the environment for 1 week prior to initiation of the experimental procedures. Animals were randomly assigned to one of four treatment groups: Saline Only Group, Alcohol Only Group, Saline+NAC, Alcohol+NAC. The repeated binge model of alcohol administration consisted of a daily intraperitoneal (I.P.) injection of a 20% (v/v) ethanol/saline solution made from 100% molecular grade absolute ethanol (Sigma–Aldrich, St. Louis, MO), and sterile isotonic saline. Mice were administered the ethanol at a dose of 2 g/kg once per day for 3 consecutive days, then allowed to rest for 4 consecutive days. After the 4 days of rest, the binge ethanol administration protocol was repeated, one I.P. injection daily for 3 consecutive days. One hour after the sixth and final injection, all groups received the stabilized, mid-shaft tibia fracture surgery, as described. All animals were weighed daily prior to injection to ensure correct dosage. Mice in the saline control groups were administered sterile isotonic saline only. Blood alcohol levels averaged approximately 200 mg/dl at the time of fracture (1 h post-injection). As post-injury drinking is a clinically relevant problem,⁸ and in order to ensure alcohol-induced oxidative stress would be present

during healing, animals continued to receive group-specific injections once daily until they were humanely euthanized at either 3, 6, or 9 days post-fracture, with the Saline Only Group receiving saline, the Alcohol Only Group receiving ethanol at 2 g/kg, the Saline+NAC Group receiving saline along with NAC at 100 mg/kg, and the Alcohol+NAC Group receiving ethanol at 2 g/kg along with NAC at 100 mg/kg.

Stabilized Tibia Fracture Model

Our fracture model is a semi-rigidly stabilized, surgical mid-shaft tibia fracture model based on the model used by Chen, et al.³⁸ and has been previously used and validated by our laboratory.³⁷ We chose semi-rigid fixation because of its clinical relevancy, as semi-rigid fixation allows healing through both endochondral and intramembranous ossification.^{1,37,38} One hour after administration of the final alcohol or saline injection, mice were given an induction dose of anesthesia (0.5–0.75 mg/kg ketamine and 0.06–0.08 mg/kg xylazine) to facilitate hair removal from the left hind limb of the animal. Mice were given 5 mg/kg prophylactic gentamicin subcutaneously and anesthetized completely with isoflurane for the duration of the procedure. Under sterile conditions, the surgery site was swabbed with povidone-iodine solution followed by 70% ethanol. A small incision was made to expose the patellar tendon and a 27-gauge needle was used to ream a hole into the medullary cavity at the proximal aspect of the tibia. A stainless-steel insect pin 0.25 mm in diameter (Fine Science Tools Inc., Foster City, CA) was inserted into the reamed hole to stabilize the tibia. A pair of angled bone scissors (Fine Science Tools) was used to surgically create a mid-diaphyseal tibial fracture. The insect pin was cut flush with the bone and the wound was sutured closed. Mice were then placed in clean cages on heating pads with free access to food and water. All animals received postoperative buprenex subcutaneously (0.05 mg/kg/q8) for pain control for 24 h post-injury.

N-Acetyl-L-Cysteine Administration

Sigma grade n-acetyl-cysteine (NAC) (>99%, Sigma–Aldrich) was diluted into solution in sterile isotonic saline and administered by once daily I.P. injections at 100 mg/kg to the animals in both the Saline + NAC Group and the Alcohol+NAC group during the fracture healing process until the animals were humanely euthanized at either 3, 6, or 9 days post-fracture.

Fracture Callus Histology

Injured and contralateral tibias were harvested from the mice 3, 6, and 9 days post-fracture and placed in 10% neutral buffered formalin for 48 h. The tibias were decalcified in 10% EDTA with agitation for 5 days, processed through a graded series of alcohol solutions and xylene, and infiltrated overnight with melted paraffin at 56–58°C. The tibias were oriented identically during paraffin embedding in order to identify mid-callus sections. Five-micrometer longitudinal sections were taken at the middle of the callus and placed onto Superfrost © Plus slides (Fisher Scientific, Pittsburgh, PA) and baked on a 60°C slide warmer overnight. Sections from each group were stained routinely with hematoxylin and eosin. Slides were scanned and uploaded to highresolution TIFF image files. ImageJ software (Public Domain, courtesy of the National Institutes of Health, Bethesda, MD) was used to quantify areas of the image files.

Western Blot Analysis

Fractured tibias were harvested from mice 3, 6, and 9 days post-fracture and snap-frozen in liquid nitrogen. Fracture callus tissue was isolated using a Dremel tool (Dremel Inc., Racine, WI) while frozen, and pulverized in RIPA lysis buffer using a freezer mill (SPEX CertiPrep Inc., Metuchen, NJ). Total protein was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Rockford, IL). Twenty microgram of total protein from each sample was resolved on 4–20% SDS–PAGE, electro-transferred to PVDF membranes, and probed with rabbit anti-mouse total FoxO (Abcam, Cambridge, MA), FoxO P-S207 (Cell Signaling, Danvers, MA), or FoxO P-S253 (Abcam). The FoxO P-S207 antibody was only used at day 6 post-fracture because the antibody has been unavailable recently, and day 6 is the time point in which we find the most drastic changes in callus histology. Our previous experiments indicate that the expression of typical housekeeping genes (GAPDH, actin, PGK-1, β -tubulin) changes throughout the course of fracture repair, and the expression of each is significantly altered by alcohol exposure. Therefore, to ensure equal loading of protein, the transferred membranes were Coomassie-stained following protein detection, and values were normalized to a 60 kD band on the stained membrane, as previously demonstrated.³⁷ Densitometric analysis was carried out utilizing Image Lab software (Bio-Rad Inc., Hercules, CA) and western blot data were presented as the densitometric ratio of target protein:Coomassie-stained band.

Statistics

Data are expressed as mean $\pm$ standard error. Statistical differences between the saline and alcohol groups for western blot analysis were calculated using Student's *t*-test. Statistical differences in callus areas between all four treatment groups were calculated by one-way ANOVA with Tukey's post-hoc testing. A *p*-value below 0.05 was considered significant.

RESULTS

Alcohol Administration Inhibited Cartilaginous Callus Formation

Figure 1A shows representative images of the fracture callus histology with hematoxylin and eosin (H&E) staining from all four treatment groups at 6 days post-fracture; Saline, Alcohol, Saline + NAC, and Alcohol + NAC. Callus histology of saline-treated mice displays the normal patterns of fracture healing through endochondral ossification, including a large external, cartilaginous callus initially formed around the fracture, with cartilaginous callus formation peaking around this time point (Fig. 1A). Additional hallmarks of endochondral ossification in the saline-treated animals include robust cartilage matrix deposition and abundant mature, hypertrophic chondrocytes within the cartilaginous callus. Illustrative examples of areas of hypertrophic chondrocytes have been denoted with arrows (Fig. 1A, arrows). These characteristics are still prevalent in the histology at 9 days post-fracture (Fig. 2A, arrows); however, there is more evidence of ossification occurring and new, woven bone being formed within the callus.

Fracture callus histology of alcohol treated animals displayed decreased formation of the cartilaginous callus, as well as suppressed hallmarks of endochondral ossification, cartilage matrix deposition, and mature hypertrophic chondrocytes (Fig. 1B).

Computer imaging software was used to quantify different callus areas. At 6 days post-fracture, alcohol significantly inhibited the formation of both the total cartilaginous callus, as shown by the decrease in the cartilaginous area (Fig. 1B, $p=.0254$), and chondrocyte maturation, as shown by the decrease in the hypertrophic chondrocyte area (Fig. 1C, $p=.0004$). Even when the area of hypertrophic chondrocytes is expressed as a percentage of the total cartilaginous area, to control for the decrease in overall area available for the forming hypertrophic chondrocytes, there was still a significant decrease in the callus tissue of alcohol-treated animals as compared to saline-treated animals (Fig. 1D, $p=.0003$).

These trends were also apparent at 9 days post-fracture (Fig. 2A). The callus tissue of alcohol-treated animals exhibited similar significant decreases in cartilaginous area (Fig. 2B, $p=.0009$) and hypertrophic chondrocyte area (Fig. 2C, $p=.0146$). However, the decreased hypertrophic chondrocyte area due to alcohol treatment was no longer statistically significant when expressed as a percentage of the total cartilaginous callus area (Fig. 2D, $p=.1289$).

Antioxidant Treatment Prevented Alcohol-Induced Callus Perturbation

In order to determine if the deleterious effects of alcohol on fracture formation could be attributed to alcohol's ability to increase oxidative stress, we administered n-acetyl-cysteine (NAC) during healing to prevent ROS accumulation. First, we saw that NAC did not affect normal fracture healing in our saline treated animals (Figs. 1A and 2A). The callus histology of animals treated with saline and NAC was normal at 6 and 9 days post-fracture, with the various callus components and all the indicative characteristics of endochondral ossification remaining similar to animals only treated with saline when viewed histologically (Figs. 1A and 2A) and when quantified (Figs. 1B–D and 2B–D).

NAC was able to prevent the disruptions in normal callus formation seen with alcohol administration. NAC treatment largely recovered proper endochondral ossification and cartilaginous callus formation in alcohol treated animals, along with the recurrence of the characteristic hallmarks of cartilage matrix deposition and mature, hypertrophic chondrocytes at 6 days post-fracture (Fig. 1A, arrows) and 9 days post-fracture (Fig. 2A, arrows). Concurrent NAC treatment during healing restored the cartilaginous callus areas and the hypertrophic chondrocyte areas back to similar levels of animals treated with only saline or saline and NAC at days 6 and 9 post-fracture (Figs. 1B–D and 2B–D).

Alcohol Administration Increased FoxO Activation Within the Fracture Callus

Next, our laboratory examined whether alcohol administration could modulate FoxO expression or activation in isolated callus tissue using western blot analysis. Total FoxO1 protein expression was increased in the fracture callus tissue of alcohol treated animals 6 days post-fracture (Fig. 4A, $p=.0045$). Alcohol-induced FoxO1 expression was not significant at 3 (Fig. 3A, $p=0.3913$) or 9 (Fig. 5, $p=0.3487$) days post-fracture.

FoxO activity is primarily modulated through post-translational modifications. FoxO becomes inactivated when phosphorylated at Serine-253 and activated when phosphorylated at Serine-207.³⁹ By using antibodies specific for phosphorylation at these particular residues, we can approximate the activation status of FoxO. We found that alcohol increased

the marker of FoxO activation (P-S207) within callus tissue 6 days post-fracture (Fig. 4B, $p = 0.0002$), while alcohol concurrently decreased the marker of FoxO inactivation (P-S253) at days 3 (Fig. 3B, $p = 0.0001$) and 6 (Fig. 4C, $p = 0.0185$) post-fracture. The decrease in the marker of FoxO inactivation (P-S253) within callus tissue of alcohol-treated animals as compared to saline-treated animals at 9 days post-fracture failed to reach significance in this study (Fig. 5B, $p = 0.0673$).

Antioxidant Treatment Abolished Markers of Alcohol-Induced FoxO Activation

In order to validate that the change in phosphorylation status of FoxO transcription factors in our model of deficient fracture healing was being caused by alcohol-induced oxidative stress, we used western blotting to examine the expression and different phosphorylation markers of FoxO in callus tissue of animals treated with n-acetyl cysteine (NAC). In the presence of NAC, alcohol administration had no effect on FoxO1 expression at 3, 6, and 9 days post-fracture in the alcohol administration group as compared to their saline cohorts (Figs. 3C, 4D, and 5C). Likewise, there was no effect of alcohol on the marker of FoxO activation, phosphorylation at Serine-207, at day 6 post-fracture with concurrent NAC administration (Fig. 4E). In the presence of NAC there also was no alcohol-induced decrease of the marker of FoxO inactivation, phosphorylation at Serine-253, at each time point during healing (Figs. 3D, 4F and G, and 5D). It is also important to note that when all four treatment groups, Saline, Alcohol, Saline + NAC, and Alcohol + NAC, were examined together, only the alcohol-treated group was significantly different from all the other groups (Fig. 4G, $p = 0.0001$). In animals receiving alcohol, NAC treatment was able to restore FoxO protein back to the levels of saline-treated animals.

DISCUSSION

There are approximately 16 million bone fractures that occur annually within the United States alone, yet, around 10% of those fractures fail to heal properly without extensive intervention contributing to a significant burden on the individual and the healthcare system as a whole.¹ These facts underscore the significance of understanding the intricate factors that contribute to improper healing, as well as elucidating any potential clinical therapy.

The underlying factors contributing to nonunion are not well understood, but our laboratory has developed a clinically relevant model of delayed union using binge alcohol administration in rodents. Previous studies into the effects of alcohol on fracture healing have utilized osteotomies with immobilization, and have focused primarily on late-stage mineralization.^{5,9,17,18} While important, these studies are limited to examining how alcohol affects osteoblast function and subsequent mineralization. Also, the studies that have investigated alcohol's effects on callus initiation and early formation concentrate on systemic and local inflammatory signaling.^{14–16}

In this study, we have created a clinically-relevant model of fracture healing that recapitulates real world parameters, including age of animals, paradigm of alcohol administration, and timing of injury. We used force to generate the fracture, and utilized intramedullary pinning to create semi-rigid fixation of the fracture. Semi-rigid fixation is especially important because a small amount of motion at the fracture site during healing is

necessary to drive cartilaginous callus formation and subsequent endochondral ossification. Rigid fixation of the fracture produces healing through intramembranous ossification, and therefore would not be an appropriate model to examine effects of treatment on external, cartilaginous callus formation. Thus, our model makes it possible to study the process through which most long bone fractures heal in humans, both at early and late stages of healing.

We have shown that episodic binge alcohol exposure in mice before and after fracture impairs the process of endochondral ossification. We see significant reductions in the formation of the cartilaginous callus and the maturation of chondrocytes in our alcohol-treated animals. Both of these reductions may speak to an impairment in mesenchymal stem cell (MSC) differentiation and chondrogenesis. Previous data from our lab have shown that alcohol disrupts gene expression regulated by canonical Wnt signaling within the fracture callus.^{37,39} The canonical Wnt signaling pathway is important during healing as it drives the differentiation of MSCs towards osteoblasts and chondrocytes that are indispensable in proper healing. To this end, Wnt signaling relies on the transcription factor β -catenin to bind to the cofactor TCF/LEF in order to up regulate genes related to chondrogenesis and osteogenesis. The family of FoxO transcription factors; however, binds to β -catenin, leading to the antagonism of Wnt-related gene induction. FoxOs are known to be activated by oxidative stress, and alcohol-abuse is a known contributor to oxidative stress in the body.¹⁹ Therefore, we hypothesized that episodic ethanol administration would lead to deficient fracture repair by activating FoxO transcription factors within the fracture callus, suppressing chondrogenesis and subsequent cartilaginous callus formation. In this study, not only did we demonstrate alcohol's suppression of fracture healing, namely through impairments in cartilaginous callus formation, but we have shown that alcohol-administration in mice was able to produce a pattern of post-translational modifications indicative of FoxO signaling activation during the healing process. We also went on to delineate that these findings were the result of alcohol-induced oxidative stress by using an antioxidant n-acetyl-cysteine (NAC) to prevent the aforementioned perturbations in callus component formation, as well as the increase in FoxO expression and markers of activation within the callus of alcohol-treated animals. This is the first report to quantify alcohol's inhibition of cartilaginous callus formation early in the healing process, as well as the first to associate these effects with FoxO activation induced by systemic oxidative stress.

Our findings in a fracture injury model support the work of other groups, which have found that activation of FoxO signaling in skeletal cells in vivo and in vitro leads to a decrease in Wnt signaling, osteoblast formation, and overall bone health.²⁵ Interestingly, there appears to be a link between aging and an increased systemic oxidative load, leading to an activation of FoxO signaling and subsequent skeletal involution,^{40,41} which serves to further highlight the importance of our work as alcohol abuse seems to be recapitulating a proposed mechanism of skeletal involution related to aging.

Alcohol consumption may have similar effects on fracture healing as diabetes, another pro-oxidative condition. Diabetes, much like alcohol abuse, has associated maladies like osteopenia, decreased bone mineral density, and impaired fracture healing.^{42,43} One proposed mechanism for the perturbed healing in diabetics is through FoxO1 stimulating

chondrocyte apoptosis.^{42,43} In our study, however, we did not observe an increase in markers of apoptosis in our days 3 and 6 six post-injury fracture callus samples (Supplemental Figure). The possible explanations for these varying results include the differences in time points following injury examined between the two studies, as well as the differences between the chronic nature of diabetes-related stress responses and the more acute stress of alcohol exposure, with their associated differences in signaling upstream of FoxO1 connected to either chronic diabetic hyperglycemia or transient alcoholic oxidative stress. Meaning that activation of FoxO could happen through different means in each model, with diabetic activation happening through suppression of Akt signaling and alcohol-associated activation happening through MST1 or JNK signaling. These findings just serve to highlight the varied roles that FoxO signaling may play based upon the sum of different intracellular cues. Perhaps in our acute alcohol-based model, FoxO1 activation disrupts MSC differentiation pathways away from chondrogenesis, rather than inducing apoptosis in differentiated chondrocytes.

One unexpected result of this study was that we did not witness significant changes in FoxO expression or markers of activation at day 9 post-fracture. As of yet, we do not completely understand why alcohol is failing to elicit much of a FoxO response at this time point. This could possibly be due to compensatory cellular mechanisms taking place after the extended alcohol treatment, or could be more likely due to the gross changes in the callus at this time. The callus by day 9 has become a lot less cellular than previous time points, and is now beginning to ossify. So at day 9 there is more mineral deposition, less active chondrocytes, and less chondrocyte maturation taking place. All of this, paired with the marked decrease in MSC differentiation at day 9, may explain why our alcohol treatment failed to elicit much of a change in FoxO compared to the saline treatment.

Since alcohol is appearing to predominantly affect cartilaginous callus formation, maybe alcohol disproportionately affects chondrogenesis and chondrocyte maturation and function. Also, it is possible that already-formed and mature, resident osteoblasts within the bone lining can function relatively undisturbed by alcohol exposure. Maybe it is only the MSCs and chondroblastic or osteoblastic precursors that bear the brunt of alcohol's deleterious effects as the cells cannot properly balance fighting an increased oxidative load while concomitantly attempting to differentiate. If alcohol is disrupting MSC differentiation during early callus formation, then it could have long lasting effects on late-stage callus formation and eventual fracture healing.

Ultimately, this study leaves more work to be done to directly connect alcohol-induced oxidative stress and FoxO signaling with decreased canonical Wnt signaling and perturbed differentiation potential of MSCs. The purpose of this study was to merely determine if alcohol-induced oxidative stress was a leading contributor to poor fracture healing in our model, and if this process could involve FoxO signaling. There are many different cell types within the fracture callus. Our study is unable to differentiate between these cell types. We next need to show alcohol's ability to increase oxidative stress in MSCs, promote the interaction between FoxOs and β -catenin, disrupt Wnt driven transcription and increase FoxO driven transcription, and disrupt normal differentiation all in MSCs. These are all necessary to prove that this is indeed a mechanism through which alcohol is having its

effects, and that these overall effects of alcohol on fracture healing act through MSCs and modulation of differentiation, and not through other, more indirect means.

For now, the findings of this study may be limited to the rodent model, as fracture healing in mice is accelerated compared to humans and rodents have growth plates that persist throughout aging. However, we have shown that episodic or binge drinking can lead to significant delays in fracture healing, which could eventually impact the treatment of patients presenting with a high blood alcohol content. Also, this work has begun to elucidate a link between alcohol-induced oxidative stress and perturbed fracture healing. Certain basal levels of ROS are important for intracellular signaling at different stages of fracture healing, so a blanket suppression of ROS during healing may not be advantageous.^{44–46} However, antioxidant therapies stand to be potentially beneficial for individuals at risk for a pathologically elevated oxidative load, such as alcohol abusers. This may not only be true for fracture repair, but could extend to a myriad of MSC therapies, as excessive oxidative stress could impair MSC function and differentiation.

Acknowledgments

Grant sponsor: National Institutes of Health; Grant numbers: NIH F31 AA021308, NIAAA R01 AA016138, NIAAA R21 AA021225, NIH T32 AA013257; Grant sponsor: Orthopaedic Research and Education Foundation Resident Clinician Scientist Training; Grant number: #12-024.

References

1. Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions. *Injury*. 2007; 38:S3–S9. [PubMed: 17920415]
2. Soffer D, Zmora O, Klausner JB, et al. Alcohol use among trauma victims admitted to a level 1 trauma center in Israel. *IMAJ*. 2006; 8:98–102. [PubMed: 16544731]
3. Chakkalakal DA. Alcohol-induced bone loss and deficient bone repair. *Alcohol Clin Exp Res*. 2005; 29:2077–2090. [PubMed: 16385177]
4. Bikle DD, Genant HK, Cann C, et al. Bone disease in alcohol abuse. *Ann Intern Med*. 1985; 103:42–48.
5. Kristensson H, Lundén A, Nilsson BE. Fracture incidence and diagnostic roentgen in alcoholics. *Acta Orthop Scand*. 1980; 51:205–207. [PubMed: 7435175]
6. Nyquist F, Berglund M, Nilsson BE, et al. Nature and healing of tibial shaft fractures in alcohol abusers. *Alcohol Alcohol*. 1997; 32:91–95. [PubMed: 9131898]
7. Levy RS, Hebert CK, Munn BG, et al. Drug and alcohol use in orthopedic trauma patients: a prospective study. *J Orthop Trauma*. 1996; 10:21–27. [PubMed: 8926551]
8. Savola O, Niemelä O, Hillbom M. Alcohol intake and the pattern of trauma in young adults and working aged people admitted after trauma. *Alcohol Alcohol*. 2005; 40:269–273. [PubMed: 15870091]
9. Jänicke-Lorenz J, Lorenz R. Alcoholism and fracture healing. A radiological study in the rat. *Arch Orthop Trauma Surg*. 1984; 103:286–289. [PubMed: 6542345]
10. Callaci JJ, Himes R, Lauing K, et al. Binge alcohol-induced bone damage is accompanied by differential expression of bone remodeling-related genes in rat vertebral bone. *Calcif Tissue Int*. 2009; 84:474–484. [PubMed: 19330277]
11. Volkmer DL, Sears B, Lauing KL, et al. Antioxidant therapy attenuates deficient bone fracture repair associated with binge alcohol exposure. *J Orthop Trauma*. 2011; 25:516–521. [PubMed: 21738068]
12. Elmali N, Ertem K, Ozen S, et al. Fracture healing and bone mass in rats fed on liquid diet containing ethanol. *Alcohol Clin Exp Res*. 2002; 26:509–513. [PubMed: 11981127]

13. Lane JM, Boskey AL, Li WKP, et al. A temporal study of collagen, proteoglycan, lipid and mineral constituents in a model of endochondral osseous repair. *Metabol Bone Bio.* 1979; 1:319–324.
14. Perrien DS, Brown EC, Fletcher TW, et al. Interleukin-1 and tumor necrosis factor antagonists attenuate ethanol-induced inhibition of bone formation in a rat model of distraction osteogenesis. *J Pharmacol Exp Ther.* 2002; 303:904–908. [PubMed: 12438508]
15. Perrien DS, Liu Z, Wahl EC, et al. Chronic ethanol exposure is associated with a local increase in TNF-alpha and decreased proliferation in the rat distraction gap. *Cytokine.* 2003; 23:179–189. [PubMed: 12967643]
16. Perrien DS, Wahl EC, Hogue WR, et al. IL-1 and TNF antagonists prevent inhibition of fracture healing by ethanol in rats. *Toxicol Sci.* 2004; 82:656–660. [PubMed: 15470231]
17. Nyquist F, Halvorsen V, Madsen JE, et al. Ethanol and its effects on fracture healing and bone mass in male rats. *Acta Orthop Scand.* 1999; 70:212–216. [PubMed: 10366927]
18. Brown EC, Perrien DS, Fletcher TW, et al. Skeletal toxicity associated with chronic ethanol exposure in a rat model using total enteral nutrition. *J Pharmacol Exp Ther.* 2002; 301:1132–1138. [PubMed: 12023547]
19. Das SK, Vasudevan DM. Alcohol-induced oxidative stress. *Life Sci.* 2007; 81:177–187. [PubMed: 17570440]
20. Sedding DG. FoxO transcription factors in oxidative stress response and ageing—a new fork on the way to longevity? *Biol Chem.* 2008; 389:279–283. [PubMed: 18208360]
21. Greer EL, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene.* 2005; 24:7410–7425. [PubMed: 16288288]
22. Calnan DR, Brunet A. The FoxO code. *Oncogene.* 2008; 27:2276–2288. [PubMed: 18391970]
23. Essers MA, Weijzen S, de Vries-Smith AM, et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J.* 2004; 23:4802–4812. [PubMed: 15538382]
24. Almeida M, Han L, Martin-Millan M, et al. Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J Biol Chem.* 2007; 282:27298–27305. [PubMed: 17623658]
25. Iyer S, Ambrogini E, Bartell SM, et al. FoxOs attenuate bone formation by suppressing Wnt signaling. *J Clin Invest.* 2013; 123:3409–3419. [PubMed: 23867625]
26. Mori R, Tanaka K, de Kerckhove M, et al. Reduced FOXO1 expression accelerates skin wound healing and attenuates scarring. *Am J Pathol.* 2014; 184:2465–2479. [PubMed: 25010393]
27. Glass DA, Karsenty G. Canonical Wnt signaling in osteoblasts is required for osteoclast differentiation. *Ann NY Acad Sci.* 2006; 1068:117–130. [PubMed: 16831912]
28. Glass DA, Karsenty G. In vivo analysis of Wnt signaling in bone. *Endocrinology.* 2007; 148:2630–2634. [PubMed: 17395705]
29. Monroe DG, McGee-Lawrence ME, Oursler MJ, et al. Update on Wnt signaling in bone cell biology and bone disease. *Gene.* 2012; 492:1–18. [PubMed: 22079544]
30. Dong YD, Soung do Y, Schwarz EM, et al. Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. *J Cell Physiol.* 2006; 208:77–86. [PubMed: 16575901]
31. Hoogeboom D, Essers MA, Polderman PE, et al. Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. *J Biol Chem.* 2008; 283:9224–9230. [PubMed: 18250171]
32. Chen JR, Shankar K, Nagarajan S, et al. Protective effects of estradiol on ethanol-induced bone loss involve inhibition of reactive oxygen species generation in osteoblasts and downstream activation of the extracellular signal-regulated kinase/signal transducer and activator of transcription 3/receptor activator of nuclear factor-kappaB ligand signaling cascade. *J Pharmacol Exp Ther.* 2008; 324:50–59. [PubMed: 17916759]
33. Chen JR, Lazarenko OP, Shankar K, et al. A role for ethanol-induced oxidative stress in controlling lineage commitment of mesenchymal stromal cells through inhibition of Wnt/beta-catenin signaling. *J Bone Miner Res.* 2010; 25:1117–1127. [PubMed: 20200986]
34. Zhou H, Shang L, Li X, et al. Resveratrol augments the canonical Wnt signaling pathway in promoting osteoblastic differentiation of multipotent mesenchymal cells. *Exp Cell Res.* 2009; 315:2953–2962. [PubMed: 19665018]

35. Essers MAG, de Vries-Smits LMM, Barker N, et al. Functional interaction between β -catenin and FOXO in oxidative stress signaling. *Science*. 2005; 308:1181–1184. [PubMed: 15905404]
36. Lauing K, Himes R, Rachwalski M, et al. Binge alcohol treatment of adolescent rats followed by alcohol abstinence is associated with site-specific differences in bone loss and incomplete recovery of bone mass and strength. *Alcohol*. 2008; 42:649–656. [PubMed: 19038696]
37. Lauing KL, Roper PM, Nauer RK, et al. Acute alcohol exposure impairs fracture healing and deregulates β -catenin signaling in the fracture callus. *Alcohol Clin Exp Res*. 2012; 36:2095–2103. [PubMed: 22691115]
38. Chen Y, Whetstone HC, Lin AC, et al. Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. *PLoS Med*. 2007; 4:1216–1229.
39. Callaci JJ, Himes R, Lauing K, et al. Long-term modulations in the vertebral transcriptome of adolescent-stage rats exposed to binge alcohol. *Alcohol Alcohol*. 2010; 45:332–346. [PubMed: 20554695]
40. Lehtinen MK, Yuan Z, Boag PR, et al. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell*. 2006; 125:987–1001. [PubMed: 16751106]
41. Hoogeboom D, Burgering BM. Should I stay or should I go: beta-catenin decides under stress. *Biochim Biophys Acta*. 2009; 1796:63–74. [PubMed: 19268509]
42. Kayal RA, Siqueira M, Alblowi J, et al. TNF-alpha mediates diabetes-enhanced chondrocyte apoptosis during fracture healing and stimulates chondrocyte apoptosis through FOXO1. *J Bone Miner Res*. 2010; 25:1604–1615. [PubMed: 20200974]
43. Ko KI, Coimbra LS, Tian C, et al. Diabetes reduces mesenchymal stem cells in fracture healing through a TNF α -mediated mechanism. *Diabetologia*. 2015; 58:633–642. [PubMed: 25563724]
44. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocr Rev*. 2010; 31:266–300. [PubMed: 20051526]
45. Zuscik MJ, Hilton MJ, Zhang X, et al. Regulation of chondrogenesis and chondrocyte differentiation by stress. *J Clin Invest*. 2008; 118:429–438. [PubMed: 18246193]
46. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. *Curr Biol*. 2014; 24:453–462.

Figure 1.

The effects of alcohol and antioxidant (NAC) treatment on callus components at day 6 post-fracture. (A) Representative H&E images of fracture calluses from each treatment group (Saline, Alcohol, Saline + NAC, and Alcohol + NAC) 6 days after fracture at 20× and 100× magnification. Lines indicate transverse fracture site; boxes indicate site of magnification; arrowheads indicate sites of hypertrophic chondrocytes. (B–D) Quantifications of (B) the total area of the cartilaginous component of the callus, (C) the total area of hypertrophic chondrocytes within the callus, and (D) the percentage of the total area of the cartilaginous component of the callus that was composed of hypertrophic chondrocytes are shown comparing all four treatment groups. Different letters denote a statistical difference between groups compared by one-way ANOVA.

Figure 2.

The effects of alcohol and antioxidant (NAC) treatment on callus components at day 9 post-fracture. (A) Representative H&E images of fracture calluses from each treatment group (Saline, Alcohol, Saline+NAC, and Alcohol+NAC) 9 days after fracture at 20× and 100× magnification. Lines indicate transverse fracture site; boxes indicate site of magnification; arrowheads indicate sites of hypertrophic chondrocytes. (B–D) Quantifications of (B) the total area of the cartilaginous component of the callus, (C) the total area of hypertrophic chondrocytes within the callus, and (D) the percentage of the total area of the cartilaginous component of the callus that was composed of hypertrophic chondrocytes are shown comparing all four treatment groups. Different letters denote a statistical difference between groups compared by one-way ANOVA.

3 Day 3

Figure 3.

FoxO protein expression and markers of inactivation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at day 3 post-fracture. Western blot of (A) total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (C) total FoxO1 with NAC; and (D) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC. Corresponding quantification is graphed underneath each blot; * $p < 0.05$ by Student's t -test.

Figure 4.

FoxO protein expression and markers of activation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at day 6 post-fracture. Western blot of (A) total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 207 (P-S207) without NAC; (C) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (D) total FoxO1 with NAC; (E) FoxO protein phosphorylated at Serine 207 (P-S207) with NAC; (F) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC; and (G) FoxO protein phosphorylated at Serine 253 (P-S253) in Saline and Alcohol groups with and without NAC. Corresponding quantification is graphed underneath each blot; different letters denote a statistical difference between groups compared by one-way ANOVA, and * $p < 0.05$ by Student's t -test.

5 Day 9

Figure 5.

FoxO protein expression and markers of inactivation in callus tissue after Saline or Alcohol treatment with or without corresponding antioxidant (NAC) treatment at day 9 post-fracture. Western blot of (A) total FoxO1 without NAC; (B) FoxO protein phosphorylated at Serine 253 (P-S253) without NAC; (C) total FoxO1 with NAC; and (D) FoxO protein phosphorylated at Serine 253 (P-S253) with NAC. Corresponding quantification is graphed underneath each blot; * $p < 0.05$ by Student's t -test.