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ACUTE ALCOHOL EXPOSURE IMPAIRS FRACTURE HEALING AND DEREGULATES β -CATENIN SIGNALING IN THE FRACTURE CALLUS

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Abstract

Background—Alcohol abuse is a risk factor for bone damage and fracture-related complications. Through precise β -catenin signaling, canonical Wnt signaling plays a key role in fracture repair by promoting the differentiation of new bone and cartilage cells. In this study, we examined the effects of alcohol on the Wnt pathway in injured bone using a murine model of alcohol-induced impaired fracture healing.

Methods—Male C57Bl/6 or TCF-transgenic mice were administered 3 daily intraperitoneal doses of alcohol or saline. One hour following the final injection, mice were subjected to a stabilized, mid-shaft tibial fracture. Injured and contralateral tibias were harvested at 6, 9, or 14 days post-fracture for analysis of biomechanical strength, callus tissue composition, and Wnt/ β -catenin signaling.

Results—Acute alcohol treatment was associated with a significant decrease in fracture callus volume, diameter, and biomechanical strength at day 14 post-fracture. Histology revealed an alcohol-related reduction in cartilage and bone formation at the fracture site, and that alcohol inhibited normal cartilage maturation. Acute alcohol exposure caused a significant 2.3-fold increase in total β -catenin protein at day 6 and a significant decrease of 53% and 56% at days 9 and 14 respectively. *LacZ* staining in β -galactosidase-expressing TCF-transgenic mice revealed spatial and quantitative differences in Wnt-specific transcriptional activation at day 6 in the alcohol group. Days 9 and 14 post-fracture showed that acute alcohol exposure decreased Wnt transcriptional activation, which correlates with the modulation of total β -catenin protein levels observed at these time points.

Conclusions—Acute alcohol exposure resulted in significant impairment of fracture callus tissue formation, perturbation of the key Wnt pathway protein β -catenin, and disruption of normal Wnt-mediated transcription. These data suggest that the canonical Wnt pathway is a target for alcohol in bone, and may partially explain why impaired fracture healing is observed in alcohol-abusing individuals.

Keywords

Acute alcohol; Wnt; β -catenin; fracture repair

Introduction

Fracture nonunion, or cessation of bone healing without bridging, occurs in 5–10% of the estimated 13 million fractures that are treated annually in the U.S. (Einhorn, 1995; Marsh, 1998; AAOS, 2008). The rate of fracture among alcoholics is up to four times higher than in non-abusers (Kristensson et al., 1980), and alcoholics frequently present with osteopenia or osteoporosis (Bikle et al., 1985; Spencer et al. 1986). Additionally, acute alcohol intoxication is demonstrated in 25–40% of patients presenting with orthopaedic trauma, highlighting the prevalence of this type of alcohol consumption and incidence of fracture injury (Levy et al., 1996; Blake et al., 1997). Numerous clinical studies have associated alcohol abuse with a significantly increased risk of developing nonunion and delayed union (Foulk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011), and several animal studies using models of alcohol exposure and bone injury report similar observations (Jänicke-Lorenz and Lorenz, 1984; Chakkalakal et al., 2005; Trevisiol et al., 2007).

Fracture repair requires the mobilization and differentiation of mesenchymal stem cells and osteoblast precursors from local and distant niches to the site of injury (Devine et al., 2002; Shen et al. 2002; Taguchi et al., 2005; Kumagai et al. 2008). The initiation of repair requires differentiation of mesenchymal stem cells into bone and cartilage-forming cells, a process that is tightly controlled by canonical Wnt/ β -catenin signaling (Day et al., 2005; Hill et al. 2005; Baksh et al., 2007). Canonical Wnt signaling has emerged as a complex pathway that is tightly regulated during bone repair and appears to control the fracture repair process (Chen et al., 2007; Komatsu et al. 2010; Huang et al. 2011). In the absence of pathway stimulation, stabilized β -catenin levels in bone tissue are kept low by a destruction complex consisting of APC, Axin, and glycogen synthase kinase 3-beta (GSK-3 β), which promotes GSK-3 β -mediated phosphorylation of β -catenin on specific residues that lead to its proteasomal degradation. During activation of the pathway, Wnt proteins bind to Frizzled/Lrp5/6 receptors on the cell membrane, causing disintegration of the destruction complex and accumulation of β -catenin in the cytosol. β -catenin then translocates to the nucleus and promotes target gene transcription by binding to T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors (reviewed in Kikuchi, 2000).

Recent studies have emphasized the importance of β -catenin and canonical Wnt signaling in osteogenesis and chondrogenesis by highlighting that precise levels of β -catenin stabilization are required during different stages of mesenchymal stem cell differentiation toward the osteoblast and chondrocyte lineage (Day et al., 2005; Silkstone et al., 2008; Miclea et al., 2009). Furthermore, deletion of β -catenin in osteoblasts or chondrocytes in mouse models of fracture repair in the tibia have been shown to cause delayed union and decreased bone and cartilage formation within the fracture callus (Chen et al., 2007, Huang et al. 2011). To date, no studies have examined whether alcohol exposure causes disruption of this pathway or β -catenin stabilization during fracture repair, which would contribute to our understanding of fracture-related complications seen in alcohol-abusing individuals.

Our lab has previously published data in rodents showing acute alcohol exposure modulates the expression of a number of genes belonging to the canonical Wnt pathway in bone tissue, including β -catenin (Himes et al., 2008; Callaci et al. 2010). Therefore, using a mouse model of acute alcohol exposure and tibial fracture, we sought to determine whether a).

acute alcohol exposure impairs normal fracture healing and b). if alcohol exposure causes disruption of Wnt/ β -catenin signaling in the fracture callus during the repair process. For the current investigation, we hypothesized that acute alcohol exposure prior to orthopaedic injury would lead to decreased bone healing and cause deregulation of β -catenin levels during fracture repair.

Materials and Methods

Acute 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 either the Saline + Fracture group or the Alcohol + Fracture group. The acute alcohol exposure utilized in all experiments consisted of a daily intraperitoneal 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/saline solution at a dose of 2 g/kg once per day for 3 consecutive days, and were weighed daily prior to injection to ensure correct dosage. Mice in the saline control groups were administered sterile isotonic saline only. One hour after the third and final injection, all mice were subjected to the stabilized tibial fracture surgery described below. Blood alcohol levels averaged approximately 200 mg/dl at the time of fracture (1 hour post-injection).

Stabilized tibial fracture creation

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 isofluorane 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. An 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 post-operative buprenex subcutaneously (0.05 mg/kg) for pain control.

Fracture callus histology

Injured and contralateral tibias were harvested from the mice 6, 9, and 14 days post-fracture and placed in 10% neutral buffered formalin for 48 hours. 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- μ m sections were 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.

Four-point biomechanical testing of fracture callus strength

Injured and contralateral tibias harvested at 14 days post-fracture were utilized for 4-point bending analysis to examine the effects of alcohol on fracture callus strength. The contralateral tibias served as the uninjured control group. The contralateral and fractured tibias were loaded into a customized 4-point bending apparatus and tested at 0.5 mm/sec using a materials testing machine (Instron Corporation, Model 5544, Canton, MA). Load-deflection curves were analyzed for maximum load (load to failure). Failure was defined as a 20% reduction in secant stiffness, or the linear portion of the load-deflection curve.

Total β -catenin protein quantification

Injured and contralateral tibias were harvested from mice 6, 9, and 14 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 lysis buffer using a freezer mill (SPEX CertiPrep Inc., Metuchen, NJ). An equivalent fragment of bone was isolated from contralateral tibias, which served as baseline levels in uninjured control tissue. Total protein was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., Rockford, IL). Fifteen μ g 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 β -catenin (Millipore, Billerica, MA). 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 is significantly altered by alcohol exposure. Therefore, to ensure equal loading of protein, the transferred membranes were Coomassie-stained following total β -catenin detection (approximately 92 kD band), and values were normalized to a 60 kD band on the stained membrane. 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 total β -catenin:Coomassie-stained band.

TCF-transgenic mouse histology

Male 6–7 week old TCF-transgenic mice (B6.Cg-Tg(BAT-lacZ)3Picc/J, Jackson Labs, Bar Harbor, ME) were subjected to the acute alcohol exposure-tibial fracture protocol described above. These mice contain a *lacZ* gene inserted downstream of 7 consensus binding sites for the T-cell Factor/Lymphoid Enhancer Factor (TCF/LEF) family of transcription factors, which are activated specifically upon nuclear (activated) β -catenin binding. This allows β -galactosidase to be expressed when β -catenin/TCF/LEF-driven transcription is activated through canonical Wnt stimulation. The injured and contralateral tibias from TCF-transgenic mice were harvested at 6, 9, and 14 days post-fracture and fixed in 10% formalin at 4°C for 24 hours. The bones were rinsed in wash buffer and briefly fixed at room temperature in 0.2% glutaraldehyde. The tibias were rinsed again in wash buffer and placed in tubes containing X-Gal staining solution, consisting of 2 mM MgCl₂, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-gal (Fermentas Inc., Glen Burnie, MD), for 36 hours at 37°C. Bones were then rinsed in wash buffer followed by decalcification at 4°C in daily changes of 10% EDTA for 5 days. The tibias were then processed for paraffin-embedding as described above and counterstained with neutral red.

Statistics

Data are expressed as mean $\pm$ standard error. Statistical differences between the saline and alcohol groups for biomechanical and micro-CT analysis were calculated using Student's *t*-test. Statistical differences in β -catenin levels between injured vs. uninjured saline and alcohol groups were calculated by one-way ANOVA with Tukey's post-hoc testing. A *p*-value below 0.05 was considered significant.

Results

Effects of Acute Alcohol on Fracture Callus Size and Volume

At the time of fracture injury, the mice averaged a blood alcohol concentration of 200 mg/dl. To first examine the effects of acute alcohol exposure on the basic parameters of fracture repair, the gross observations of the callus tissue formed in each treatment group at day 14 post-fracture were recorded (Figure 1). Using digital calipers to measure external callus diameter, the alcohol-treated group displayed significantly decreased maximum callus diameter as compared to the saline-treated group. Quantitative analysis of newly formed callus tissue utilizing high-resolution micro-computed tomography (μ CT) imaging revealed that alcohol-treated mice also display a significant 47% reduction in the volume of callus tissue formed at day 14 post-injury compared to the saline group.

Effects of Acute Alcohol on Fracture Callus Tissue Composition

To investigate the effects of alcohol exposure on callus tissue formation and composition histologically, we examined H & E stained sections of fracture callus at 6, 9, and 14 days post-injury. As previously shown in stabilized, mid-diaphyseal mouse tibial fracture models, these time points represent the anabolic phase of external fracture callus formation, in which cartilage formation peaks at day 9, and the overall callus mass is greatest by day 14 (Hiltunen et al., 1993; Le et al. 2001).

As shown in the saline-fracture group in Figure 2A, there is abundant cartilage formation in the well-developed external callus (arrow) surrounding the fracture site (indicated by a black line) at post-fracture day 6. By day 9 post-fracture (Figure 2B), there is an increase in the amount of cartilage present, and hypertrophic chondrocytes are visible near the periosteal callus (inset) with associated zones of endochondral bone formation (arrow). The periosteal and endosteal callus compartments are clearly defined. By day 14 (Figure 2C), the external callus has increased in size and the cartilaginous matrix of the callus has undergone mineralization, as denoted by the presence of new woven bone. Foci of hypertrophic cartilage remain (arrow), revealing that the newly formed bone present at day 14 post-fracture was created by endochondral ossification.

In contrast to the saline group, callus size does not increase in the alcohol group between days 6 and 9, as seen in Figure 2D–E. Minimal cartilage and bone in the external callus has formed through day 14 (arrows). The periosteal callus at day 9 is not clearly defined, and appears to be replaced by immature, myofibroblastic tissue (inset, Figure 2E). The chondrocytes in the limited amount of hyaline cartilage at day 9 possess an immature and chondroblastic phenotype rather than hypertrophic. Bone formation in the alcohol group at post-fracture day 14 appears predominantly periosteal-derived (Figure 2F, arrow). Histological evidence of endochondral bone formation is absent, as indicated by the lack of cartilage and hypertrophic chondrocytes in the external callus. Minimal effects on the formation of the endosteal callus at each time point were observed.

Effects of Acute Alcohol Exposure on Biomechanical Strength of Fracture Callus Tissue

The four-point bending test is a commonly used method to assess the integrity of fracture healing in long bones (adapted from Hiltunen et al., 1993). Therefore, we tested whether the alcohol-induced changes seen in callus volume and tissue composition result in functional deficits by measuring the biomechanical strength of calluses at day 14 post-fracture. Utilizing a customized four-point bending apparatus, we observed a significant 32% reduction in the maximal load sustained by injured tibias harvested from alcohol-treated mice as compared to tibias from the saline control group (Figure 3A). We have previously established that the acute alcohol paradigm utilized in these experiments causes significant

bone loss and decreased biomechanical strength of intact rat vertebrae and tibiae (Callaci et al., 2004; Lauring et al., 2008). Therefore, we investigated whether acute alcohol treatment causes bone loss in uninjured mouse tibias by testing the contralateral tibias in the mice from each group, and found there was a significant 17% reduction in maximal load attained in the alcohol-treated group (Figure 3B).

Acute Alcohol Effects on β -catenin expression in the Fracture Callus

Due to the significance of Wnt/ β -catenin signaling in normal fracture repair, we investigated whether acute alcohol exposure disrupts the precise regulation of β -catenin levels during fracture healing. Fracture calluses from saline- or alcohol-treated mice were isolated at each time point and total β -catenin protein levels in the fracture callus lysates were examined by western blot. A representative western blot of β -catenin protein expression during fracture repair in saline or alcohol-treated mice is shown in Figure 4A. Acute alcohol exposure significantly increased β -catenin protein levels by 2.3-fold at day 6 post-fracture as compared to saline treated controls (Figure 4B). At day 9 post-fracture in the alcohol group, the levels of total β -catenin are decreased by 53% compared to levels observed in the saline group (Figure 4C). This alcohol-related decrease in β -catenin levels persists until day 14 post-fracture in which the levels of β -catenin are significantly decreased by 56% compared to saline fractured levels (Figure 4D). Nearly undetectable β -catenin levels were observed in the contralateral tibias at each time point, with no significant differences noted between alcohol and saline-treated groups.

Effects of Acute Alcohol on Wnt-Specific Transcriptional Activation in the Fracture Callus

Since β -catenin serves as the transcriptional coactivator of the canonical Wnt pathway, we sought to determine whether alcohol-induced modulation of β -catenin levels subsequently leads to changes in canonical Wnt transcriptional activation. In order to test this, we examined histological sections of fracture callus from TCF-transgenic mice (Jackson Labs, Bar Harbor, ME) that express β -galactosidase in the presence of β -catenin/TCF-driven transcription, which occurs predominantly through Wnt stimulation. Figure 5A–C demonstrates the temporal changes in Wnt transcription throughout healing in the saline-treated group. At day 6, positive *LacZ* staining is present within the cartilage most distal to the fracture site, consistent with activated β -catenin/TCF transcription within pre-hypertrophic chondrocytes and immature osteoblasts (arrow). At day 9 post-fracture, the *LacZ* staining in the saline group is robust in the trabeculae of new woven bone and in areas surrounding the hypertrophic cartilage (arrow), indicative of actively mineralizing osteoblasts replacing the cartilaginous matrix with bone. By day 14, *lacZ* staining is still abundant in the periosteal callus and in the bony callus tissue being formed furthest from the fracture site (arrows).

As seen in Figure 5D–F, there are differences in the spatial orientation and intensity of *LacZ* staining in the alcohol group as compared to saline control calluses. At day 6, Strong *LacZ* staining appears mainly concentrated in the marrow cavity adjacent to the fracture site (arrows), with a small amount of staining present in the myofibroblastic, undifferentiated tissue of the external callus. By day 9, there is a significant decrease in *LacZ* staining intensity (arrow) and cartilage formation. Similarly, the positive staining at day 14-post fracture in the alcohol group is almost entirely absent with the exception of a few foci in the most peripheral bone tissue in the external callus (arrow).

Discussion

In this study, we show that acute alcohol exposure has a deleterious effect on fracture repair in mice as demonstrated by the reduction in callus size, biomechanical strength, and

alteration in callus tissue composition at the injury site. These effects persist up to 14 days post-fracture. These findings parallel a concomitant deregulation of protein levels of a key canonical Wnt signaling molecule, β -catenin, as well as β -catenin/T-cell factor (TCF) transcriptional activation. Numerous clinical studies have associated alcohol abuse with an increased risk of sustaining an orthopaedic injury and with decreased healing potential (Fouk and Szabo, 1995; Perlman and Thordarson, 1999; Mathog et al., 2000; Williams et al., 2008; Duckworth et al., 2011), and many animal studies have demonstrated alcohol-induced bone damage and impaired healing utilizing a chronic exposure model (Jänicke-Lorenz and Lorenz, 1984; Chakkalakal et al., 2005; Trevisiol et al., 2007). However, few studies to date have investigated a molecular target of alcohol during repair, and more specifically, following acute alcohol exposure. The identification of β -catenin signaling and Wnt transcriptional activation in this study as significant targets of alcohol exposure in healing bone tissue supports our previous findings in uninjured bone, in which over 40 genes belonging to the canonical Wnt pathway were modulated following acute alcohol exposure (Himes et al., 2008).

Our gross observations and histological data show that alcohol exposure is most inhibitory to the formation of the external callus tissue and the endochondral ossification process. Not surprisingly, the alcohol-related decrease in callus tissue formation resulted in a reduction in load-bearing properties of day 14 fracture calluses as demonstrated by the biomechanical 4-point bending test. Acute alcohol exposure had a striking effect on cartilage formation compared to the saline groups at days 6 and 9 post-fracture, and resulted in the appearance of immature fibrous tissue rather than mature, hypertrophic cartilage. These observations parallel similar effects seen on external callus cartilage formation utilizing a rat model of acute binge alcohol exposure and femoral fracture repair established in our laboratory (Volkmer et al. 2011). Though we expected to see an overall decrease in bone and cartilage due to the importance of Wnt/ β -catenin signaling in osteoblast and chondrocyte formation, these results were particularly remarkable because the duration of alcohol exposure in this study was much shorter compared to previous studies reporting similar effects on external callus formation and fibrous tissue proliferation following chronic alcohol exposure (Elmali et al., 2002; Chakkalakal et al., 2005; Trevisiol et al., 2007).

Some bone and cartilage formation does occur in the alcohol group, indicating that this model of acute alcohol exposure does not completely inhibit fracture repair. We do not have functional or biological data extending further than 14 days post-injury, and though we speculate that while this brief alcohol exposure prior to injury does not cause permanent healing defects, it is sufficient to delay the crucial and tightly regulated early phases of fracture repair. In addition, the acute alcohol exposure utilized in this study does not fully represent a clinically prevalent situation in which injured patients presenting with acute alcohol intoxication are likely not first-time drinkers, nor are likely to cease drinking during the healing process. Nonetheless, our data indicate that a short exposure to high levels of alcohol prior to orthopaedic injury has the capacity to initiate a cascade of cellular events that lead to fracture repair deficiencies up to 2 weeks post-injury.

The formation of the external callus is heavily dependent upon the recruitment and differentiation of mesenchymal stem cells to the fracture site (Devine et al., 2002; Shen et al., 2002; Taguchi et al., 2005; Kumagai et al., 2008). A key regulatory pathway involved in mesenchymal stem cell differentiation into cartilage and bone is the Wnt/ β -catenin pathway. The precise regulation of β -catenin levels during early fracture repair has been shown to be essential for fracture union by controlling cartilage and bone formation within the external callus tissue (Chen et al., 2007; Huang et al., 2011). Deletion of β -catenin only in committed osteoblasts during fracture repair inhibits external callus mineralization, indicating that the osteoblast-specific regulation of β -catenin is required for fracture union and callus bone

formation (Chen et al., 2007). The controlled timing of Wnt/ β -catenin signaling activation is also required for endochondral ossification and chondrocyte maturation, and deletion of Wnt pathway proteins or ectopic expression of Wnt/ β -catenin signaling in chondrocytes inhibits these processes (Tamamura et al., 2005; Zhong et al., 2006; Chen et al., 2008).

In our acute alcohol exposure model, we observe a 2.3-fold increase in β -catenin levels in the alcohol group at day 6 compared to the saline group, while the levels at day 9 and 14 in the alcohol group are 53% and 56% lower, respectively. Peak β -catenin levels in the saline group are observed at day 9 post-fracture, when endochondral ossification has initiated, osteoblasts are actively recruited, and chondrocyte maturation and hypertrophy is evident. Alcohol exposure prior to injury shifts the peak expression of β -catenin to an earlier time point, day 6, which is accompanied by prolonged decreases in cartilage formation with minimal chondrocyte hypertrophy and the appearance of highly cellular, immature fibrous tissue in place of cartilage. These changes persist until day 14, where we observe no evidence of endochondral bone formation. This suggests that one possible mechanism underlying these observations is that the alcohol-related deregulation of β -catenin levels occurs at a critical developmental stage of fracture healing and external callus formation. Aberrant activation of Wnt/ β -catenin signaling is known to be involved in the development of fibrosis (reviewed in Lam and Gottardi, 2011), and may link our observations of the development of nonspecific fibrous tissue in the callus at day 9 in the alcohol group with deregulation of β -catenin levels. As mentioned above, previous reports have established the requirement of Wnt/ β -catenin signaling during chondrocyte maturation and subsequent endochondral ossification during development and repair (Kitagaki et al., 2003; Tamamura et al., 2005; Chen et al. 2008; Huang et al., 2011), which support that the alcohol-related inhibition of endochondral bone formation process at these time points may be directly attributed to the deregulation of β -catenin levels.

The TCF-transgenic mouse model provides further evidence that the canonical Wnt pathway is a significant target of alcohol in healing bone. This mouse model allows the expression of β -galactosidase in the presence of activated T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors by β -catenin binding, which is a necessary event of canonical Wnt pathway activation. Although not completely exclusive to canonical Wnt transcriptional activation, this mouse model is widely used to investigate the involvement of canonical Wnt signaling in various tissues. Chen et al. (2007) has previously characterized the expression of Wnt-specific transcriptional activation during normal fracture repair in the mouse, and similarly to their observations, we detect strong *LacZ* staining in the saline group at days 6 and 9 in areas consistent with the maturation of osteoblast precursors and hypertrophic chondrocytes. This is associated with peak β -catenin expression during the anabolic phase of external callus formation.

In contrast, the alcohol group displays striking spatial and quantitative changes in Wnt transcriptional activity within the fracture callus. Theoretically, changes in β -catenin protein expression, if relevant to the canonical Wnt pathway, should lead to changes in β -catenin/TCF-mediated transcriptional activity. At day 6 post-fracture in the alcohol group, *LacZ* staining is strongest in the bone marrow near the fracture site rather than in the external callus tissue, which correlates to the significant increase in β -catenin protein levels at this time point compared to saline-treated controls. This Wnt transcriptional deregulation in the bone marrow provides further evidence that a deregulation of Wnt/ β -catenin signaling may contribute to the appearance of immature, fibrous tissue by day 9 post-fracture instead of normal hyaline cartilage. Ultimately, these experiments demonstrate that in addition to deregulating β -catenin protein levels, acute alcohol exposure subsequently caused deregulation of transcriptional activation of the Wnt pathway during critical stages of

fracture callus formation, which can then lead to alterations in the expression of Wnt target genes that are necessary for fracture repair.

We cannot rule out the possibility that other pathways may be activating β -catenin. Some studies have shown that β -catenin can also be activated and translocated to the nucleus in a Wnt-independent manner following stimulation of growth factor receptors and tyrosine kinase activation (Haraguchi et al. 2004, Ji et al. 2009, Schramm et al. 2011). Others have observed that osteoblasts can form from mesenchymal stem cells via β -catenin/TCF interaction, yet this interaction is not stimulated by Wnt signaling (Qiang et al., 2009). These data provide an alternative mechanism for Wnt-independent β -catenin activation, however, previous fracture models utilizing various *in vivo* methods such as antibody-mediated inhibition of endogenous canonical Wnt antagonists, knockout of the essential Wnt coreceptor Lrp5, or utilizing lithium chloride to inhibit GSK-3 β have supported that Wnt/ β -catenin signaling predominates over other pathways in bone during fracture repair and is a central regulator of bone healing (Chen et al., 2007; Gaur et al., 2009; Komatsu et al., 2010; Ominsky et al., 2010). Lastly, in order to completely assess the effects of alcohol on activated β -catenin, nuclear levels must be specifically measured; however the Wnt-related transcriptional activation data provided by the TCF-transgenic mice support our observations that acute alcohol exposure can disrupt nuclear β -catenin/TCF signaling activity.

In summary, these data provide valuable insight into the molecular effects of alcohol during bone repair, and allow further research to be conducted on therapies to prevent or reverse alcohol-induced deregulation of Wnt/ β -catenin signaling in alcohol-abusing patients sustaining an orthopaedic injury.

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DAY 14 POST-FRACTURE	<u>Saline-Treated</u>	<u>Alcohol-Treated</u>
Callus Diameter	3.76 ± 0.42 mm	2.77 ± 0.40 mm *
Callus Volume	3885.62 ± 1126.9 mm ³	2071.40 ± 299.3 mm ³ *

Figure 1. Binge alcohol effects on fracture callus size and volume

Average fracture callus diameter (n=12/group) and volume (n=5/group) are decreased following binge alcohol treatment. Volume was measured using micro-computerized tomography image software. Representative images of post-fracture day 14 calluses from each treatment group. The black arrows denote site of fracture and surrounding callus tissue.

* $p < 0.05$, Saline-Treated vs. Alcohol-Treated, Student's t -test.

Figure 2. Histological analysis of binge alcohol treatment on fracture callus tissue composition
Representative photomicrographs of H&E stained callus sections from each treatment group at 6, 9, and 14 days post fracture (32 $\times$ magnification). The black line indicates site of fracture. In the Saline + Fracture group, calluses demonstrate abundant cartilage formation in the external callus (A, arrow) and hypertrophic chondrocytes and endochondral ossification activity (B–C, arrows/inset). In the Alcohol + Fracture group, calluses show a lack of cartilage formation (D–E, arrows), the appearance of immature fibroblastic tissue in place of cartilage (E, inset), and periosteal bone formation rather than endochondral ossification activity (F, arrow).

Figure 3. Binge alcohol effects on callus biomechanics 2 weeks post injury

Fracture calluses (A) and contralateral tibias (B) from alcohol-treated mice 14 days post-fracture show significantly reduced bending strength compared to the fracture calluses and contralateral tibias from saline-treated mice. N=14–16/treatment group. * $p < 0.05$, Student's t -test.

Figure 4. Effects of binge alcohol on total β -catenin levels in the fracture callus

(A) Representative western blot for total β -catenin protein levels in fracture callus lysates at each timepoint. To ensure equal loading, blots were Coomassie-stained after β -catenin detection and normalized to a 60 kD band. Data are presented as the densitometric ratio of total β -catenin/Commassie-stained band. Alcohol causes a significant increase in total β -catenin protein levels at day 6 (B), followed by a marked reduction in β -catenin levels at days 9 and 14 post-fracture (C–D). Groups not sharing a letter are significant, $p < 0.05$ using one-way ANOVA and Tukey's multiple comparison procedure.

Figure 5. Binge alcohol effects on Wnt-specific transcriptional activation in the fracture callus
Representative callus sections counterstained with neutral red from TCF-transgenic mice expressing *lacZ* are shown for each time point (32× magnification). Arrows in the Saline + Fracture group (A–C) denote abundant Wnt transcriptional activation in prehypertrophic chondrocytes and areas of active endochondral ossification. Arrows in post-fracture day 6 in the Alcohol + Fracture group (D) show a concentration of Wnt transcriptional activation in the medullary canal. Days 9–14 post-fracture show a reduction in the overall intensity of positive *lacZ* staining in the callus tissue (E–F, arrows).