Antioxidant Therapy Attenuates Deficient Bone Fracture Repair Associated With Binge Alcohol Exposure

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Objectives: Alcohol consumption is a known risk factor for traumatic injuries of all types and has been shown to produce detrimental effects on bone metabolism. Although the mechanisms responsible for these detrimental effects are not well characterized, oxidative stress from alcohol exposure appears to play a central role. This study was designed to examine the effect of a short-term binge alcohol consumption pattern on fracture repair and the effect of an antioxidant, N-acetylcysteine, on fracture healing after binge alcohol consumption.

Methods: One hundred forty-four adult male Sprague-Dawley rats underwent unilateral closed femur fracture after injection of either saline or alcohol to simulate a binge alcohol cycle. Animals in the antioxidant treatment group received daily N-acetylcysteine after fracture. Femurs were harvested at 1, 2, 4, and 6 weeks after injury and underwent biomechanical testing and histologic analysis.

Results: Binge alcohol administration was associated with significant decreases in biomechanical strength at 1- and 2-week time points with a trend toward decreased strength at 4- and 6-week time points as well. Alcohol-treated animals had less cartilage component within the fracture callus and healed primarily by intramembranous ossification. Administration of N-acetylcysteine in alcohol-treated animals improved biomechanical strength to levels comparable to the control animals and was associated with increased endochondral ossification.

Conclusions: Our results indicate that binge alcohol alters the quality of fracture healing after a traumatic injury and that concurrent administration of an antioxidant is able to reverse these effects.

Key Words: fracture, binge alcohol, antioxidant, canonical Wnt pathway

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INTRODUCTION

Alcohol intoxication increases the risk of traumatic injury[1,2] with 25% to 35% of nonfatal injuries associated with alcohol use.[3] Low-energy injuries such as hip fractures can also be associated with alcohol use.[4] Motor vehicle accidents that result from alcohol use cost the American public an estimated $114.3 billion annually.[5] The pattern of alcohol consumption most often associated with traumatic injury is binge drinking.[6] Patients who abuse alcohol are at higher risk for complications after fracture including nonunion.[7] In rodents, alcohol exposure is associated with impaired osteoinduction and impaired healing of surgically induced fractures,[8,9] decreases in bone mineral density, biomechanical strength,[10,11] and decreased serum levels of osteocalcin.[12]

The canonical Wnt signaling pathway plays a pivotal role in both the maintenance of bone mass and normal fracture repair.[13,14] Our laboratory has shown that alcohol exposure decreases the expression of several key genes associated with the canonical pathway and its downstream transcriptional targets[15]; however, the mechanism(s) underlying this effect are not currently known. One possible mechanism may be related to alcohol-induced oxidative stress. Studies have shown that binge alcohol exposure induces the formation of reactive oxygen species through several mechanisms,[16] and accumulation of oxygen radicals has been shown to downregulate canonical Wnt pathway activity.[17,18] Recent clinical studies found that postmenopausal women with osteoporosis demonstrated lower levels of antioxidant enzymes and higher levels of plasma malondialdehyde, a biomarker for oxidative stress.[19] Taken together, these findings suggest that alcohol-induced deficient fracture repair may be caused by an increased state of systemic oxidative stress inhibiting Wnt signaling activity required for normal bone healing.

Based on this information, we developed a novel rodent model of acute binge alcohol exposure followed by traumatic orthopaedic injury to test the hypothesis that alcohol-induced inhibition of bone fracture healing was related in part to increased systemic oxidative stress. N-acetylcysteine (NAC) is a commonly studied and clinically used antioxidant[20] shown to

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have protective effects on pulmonary function in rats after femur fracture and to reduce oxidative stress in rats exposed to chronic alcohol.[21,22] To our knowledge, there have been no studies examining the effect of binge alcohol consumption on the healing of bone fracture injuries or the effect of antioxidant therapy on the repair process. We show evidence here that exposure to alcohol before orthopaedic trauma reduces fracture callus bridging strength and inhibits normal endochondral bone formation in the callus, whereas NAC treatment produces a beneficial effect on fracture callus strength and bone deposition compromised by prior alcohol exposure.

MATERIALS AND METHODS

Binge Alcohol Treatment Paradigm

This investigation received approval from the Loyola University Institutional Animal Care and Use Committee. Adult male Sprague-Dawley rats obtained at 16 weeks of age were randomly assigned to one of three treatment groups: injury only (I), injury with binge alcohol exposure (IA), or injury with binge alcohol exposure and N-acetylcysteine treatment (IAN). Ten animals per group were used based on a power analysis performed using preliminary fracture callus biomechanical strength data (not shown). Alcohol was administered by a single daily intraperitoneal (IP) injection of a 20% (vol/vol) ethanol/saline solution at a dose of 3 g/kg chosen to achieve peak blood alcohol levels of approximately 300 mg/dL[23] and a blood alcohol level of 200 mg/dL at the time of injury. Control animals were given an IP injection of an equal volume of sterile isotonic saline at the time of alcohol group injections. Alcohol or saline injections were given starting at 9:00 AM 3 consecutive days per week for a total of 2 weeks. No IP injections were given during the intervening 4 days between binge cycles.

Surgical Procedure

One hour after the last alcohol or saline injection, animals were anesthetized (ketamine: 90 mg/kg, xylazine: 10 mg/kg IP), prepped for surgery, and given gentamicin (5 mg/kg subcutaneously). An anterior incision was made over the left knee and a medial parapatellar approach to the distal femur was performed with the patella dislocated laterally. A 1.25-mm Kirschner wire (Synthes, West Chester, PA) was passed retrograde the length of the femoral canal to the level of the greater trochanter, and the distal end was cut flush with the femoral condyles. The patella was reduced, and the extensor mechanism and the skin were repaired with interrupted 4-0 Vicryl suture. Unilateral closed femur fractures were created using a blunt three-point guillotine device[24] consisting of a platform of two pins placed approximately 15 mm apart and a blunt point attached to a sliding rod aligned to drop between the two pins. The excursion of the sliding blunt device was adjusted to 5 mm. The device was driven by dropping a 550-g weight from a height of 20 cm. After injury, animals were resuscitated with 5 mL warmed normal saline IP and returned to home cages. Buprenorphine (0.02 mg/kg subcutaneously every 8 hours) was administered for pain control for the first 24 hours postoperatively and every 8 hours thereafter as required.

Postoperative Protocol

On postoperative Day 1, animals in the IAN groups received N-acetylcysteine (400 mg/kg IP), and animals in the remaining groups received 5 mL sterile saline IP. Animals in IAN groups received N-acetylcysteine (10 mg/kg) by IP injection daily 5 days per week for 1 week (1-week group) or 2 weeks (2-, 4-, and 6-week groups) starting on postoperative Day 2; control animals received matched volume saline IP. At 1, 2, 4, and 6 weeks postinjury, animals were euthanized and both femurs were harvested through a lateral approach with the contralateral femur of Group I used as an uninjured control. Femurs were stored at –20°C for biomechanical testing or fixed in 10% formalin for histologic analysis.

Biomechanical Testing

Femoral heads were removed to allow for better placement within the testing device and the intramedullary Kirschner wire was removed. Biomechanical testing was carried out using a four-point bending device. Biomechanical analysis was carried out using an Instron biomaterials testing device (Model 5544; Norwood, MA) with the testing protocol consisting of a constant extension of 0.5 mm/min to produce a compressive force across the fracture callus. Fracture callus biomechanical failure was defined as a drop of 20% or greater in the amount of compressive force supported by the callus. Femurs were visually inspected at the time of testing to ensure material failure.

Histologic Analysis

Fixed callus specimens were decalcified in 4% formic acid solution (Cal-Ex II fixative/decalcifier; Fisher Scientific, Pittsburgh, PA) for 14 days, embedded in paraffin, sectioned along the longitudinal axis of the femoral shaft, and stained with hematoxylin and eosin. Callus histology was visualized using a Leica MZ Apo Stereomicroscope (Leica Inc, Bannockburn, IL) and images were captured using a (Nikon D80 digital camera system, Melville, NY).

Statistical Analysis

For each outcome parameter, groups were compared at each time point using a one-way analysis of variance with Tukey post hoc test. Significance was considered for $P < 0.05$.

RESULTS

Fracture Callus Biomechanics

One-Week Group

Binge alcohol exposure had a significant effect on fracture callus strength at 1 week postinjury (Fig. 1A) with maximum load sustained by callus tissue decreasing from 119.5 N in Group I to 23.4 N in Group IA (79% decrease, $P < 0.05$). Treatment with NAC after injury prevented this alcohol-related decrease in fracture callus strength. Group IAN was significantly stronger than Group IA with a 4.6-fold increase in maximum load ($P < 0.05$). Alcohol exposure also was also associated with changes in the material properties of the fracture callus (Fig 1B) with mean callus stiffness decreasing by 80% compared with callus from saline-treated

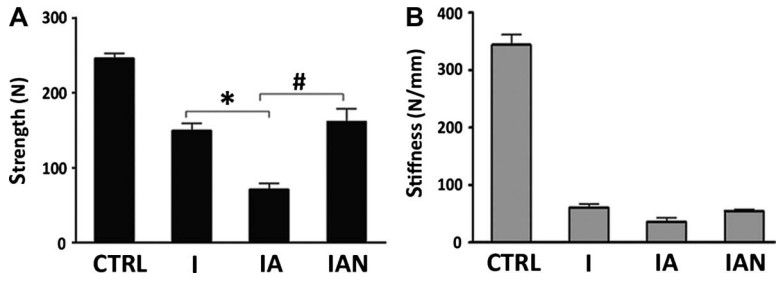

FIGURE 1. Binge alcohol and antioxidant effects on callus biomechanics at 1 week postinjury. (A) Bending strength; (B) stiffness. Treatment groups: injury, injury without alcohol; injury + alcohol, injury with binge alcohol; injury + alcohol + NAC, injury with binge alcohol and N-acetylcysteine (NAC) treatment. The alcohol-treated groups showed significantly less strength and stiffness than both the nonalcohol groups and the alcohol with NAC groups. Data were analyzed by one-way analysis of variance with Tukey post hoc test and significance considered for $P < 0.05$.

animals ($P < 0.05$). Administration of NAC also resulted in a statistically significant increase in callus stiffness in alcohol-treated animals ($P < 0.05$).

Two-Week Group

Binge alcohol continued to have detrimental effects on fracture callus strength 2 weeks after fracture injury (Fig. 2A). A 53% reduction in callus strength was observed between Group I and Group IA ($P < 0.05$). Administration of NAC was again associated with an attenuation of alcohol-related effects on callus strength with a 128% increase in strength in Group IAN compared with Group IA ($P < 0.05$). Binge alcohol treatment also continued to significantly decrease callus stiffness 2 weeks after injury (Fig 2B) (Group I vs IA, $P < 0.05$). No significant effect of NAC treatment on callus stiffness in alcohol-treated animals was observed at this time point.

Four- and Six-Week Groups

Data generated at 4 and 6 weeks after injury demonstrate continued fracture injury healing with increasing callus strength and stiffness observed at each time point in Group I (data not shown). No significant effects were noted at 4 or 6 weeks after injury in terms of biomechanical parameters between any of the treatment groups in comparison to control animals.

Fracture Callus Histology

Group I

At 1 week postinjury, a well-developed cartilaginous external and periosteal callus was present (Fig. 3A). At 2 weeks postinjury, hypertrophic chondrocytes were present throughout the external callus with associated endochondral ossification activity. By 4 weeks postinjury, the cartilaginous component of the external callus was largely replaced by woven bone. Focal areas of remodeling activity were also observed in the periosteal callus.

Group IA

At 1 week postinjury, a highly cellular external callus composed of immature chondroblast-like cells with minimal matrix deposition was present with notable deposition of collagen fibers. Minimal effects on periosteal callus development were observed (Fig. 3B). At 2 weeks postinjury, external callus tissue remained immature in nature, highly cellular with continued evidence of collagen deposition. Small focal areas of hyaline cartilage deposition were observed without evidence of hypertrophic chondrocytes or endochondral bone formation. At 4 weeks postinjury, external callus tissue remained immature and fibrous in nature; however, some focal areas of cartilage tissue and endochondral bone formation were now present.

Group IAN

At 1 week postinjury, a well-developed periosteal and cartilaginous external callus was present, similar to control animals (Fig. 3C). At 2 weeks after injury, hypertrophic chondrocytes and robust endochondral bone formation was observed. By 4 weeks postinjury, the cartilaginous external callus was largely replaced by woven bone; remodeling activity producing compact lamellar bone was also observed. Similar trends in callus development were seen in all groups at 6 weeks after injury (data not shown).

DISCUSSION

Excessive alcohol consumption has detrimental effects on bone health,[8,9,11,15,19] including a higher risk for complications after fracture injury, including nonunion.[7] The mechanisms of alcohol-deficient fracture healing are not well characterized. In the current investigation, we used a clinically relevant binge alcohol exposure model in rats to examine the effects of alcohol on fracture repair after trauma-induced

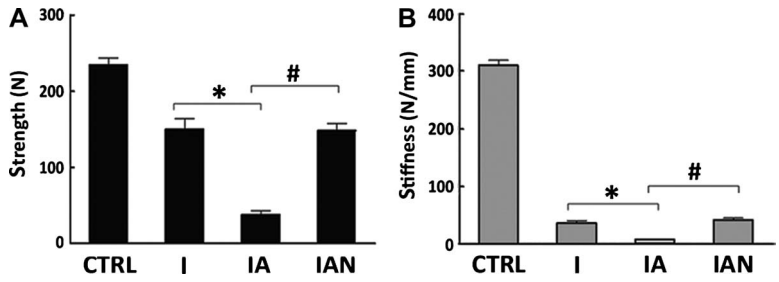

FIGURE 2. Binge alcohol and antioxidant effects on callus biomechanics at 2 weeks postinjury. (A) Bending strength; (B) stiffness. Treatment groups: injury, injury without alcohol; injury + alcohol, injury with binge alcohol; injury + alcohol + NAC, injury with binge alcohol and N-acetylcysteine (NAC) treatment. The alcohol-treated group showed significantly less strength than both the nonalcohol group and the alcohol with NAC group but no significant difference in stiffness. Data were analyzed by one-way analysis of variance with Tukey post hoc test and significance considered for $P < 0.05$.

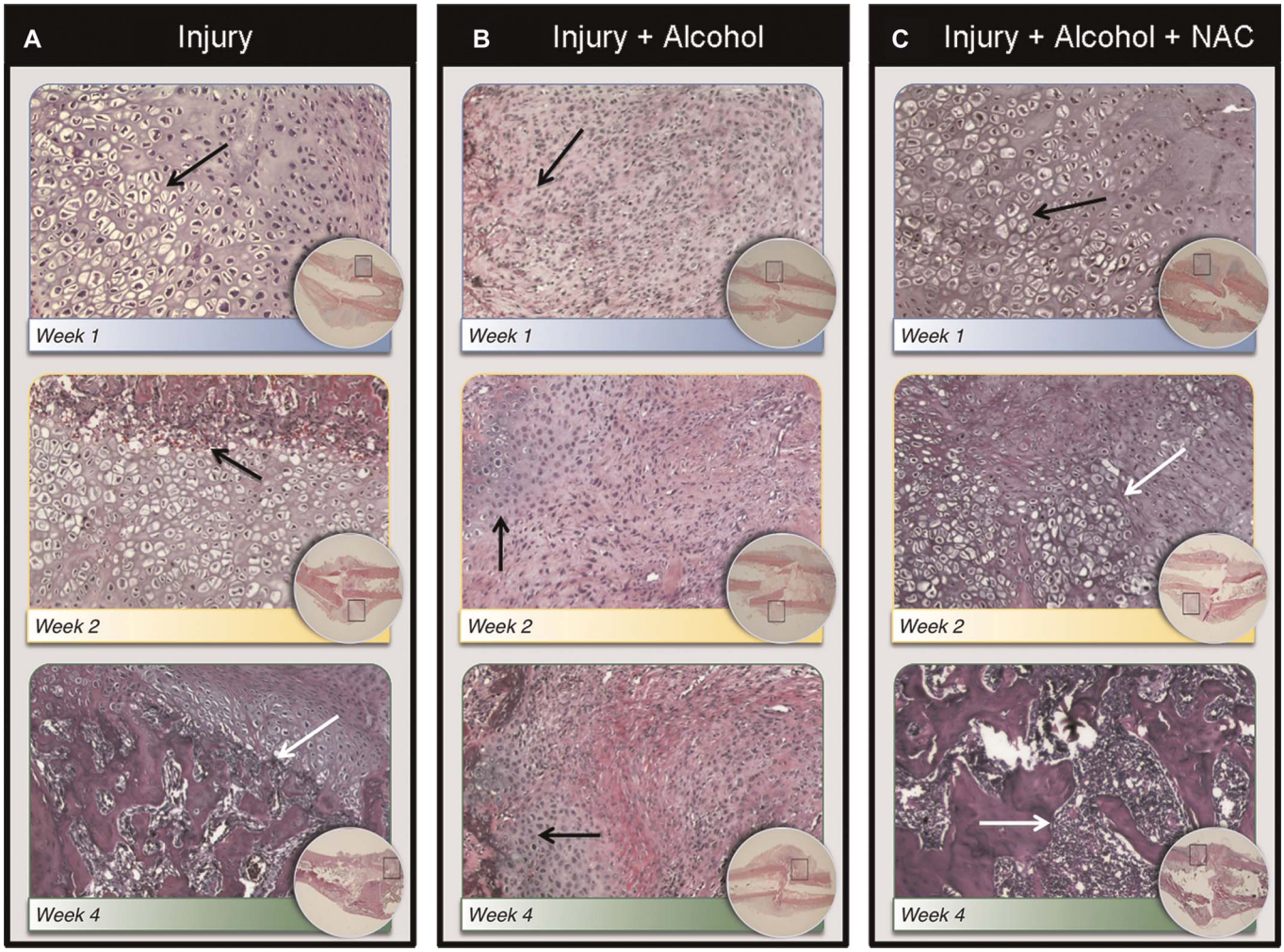

FIGURE 3. Binge alcohol and antioxidant effects on fracture callus structure and composition. Slides were stained with hematoxylin and eosin. Low-magnification images are shown as insets in each treatment group with areas of higher magnification indicated by boxed outline. Calluses in injury groups (A) showed mature and hypertrophic chondrocytes at Week 1 (black arrow), endochondral ossification activity and appearance of woven bone at 2 weeks (black arrow), and remodeling activity by 4 weeks (white arrow). Calluses in the injury + alcohol group (B) showed a fibrous callus with immature chondrocytes at Week 1 (black arrow), continuing fibrous callus with the appearance of some mature chondrocytes without significant endochondral ossification activity at 2 weeks (black arrow), and delayed ossification at 4 weeks (black arrow). Samples in the injury + alcohol + NAC group (C) showed normal early cartilaginous callus formation in Weeks 1 and 2 (black arrows) and endochondral ossification at 2 weeks (black arrow) with active bone remodeling at 4 weeks (white arrow). Samples at 6 weeks showed similar trends (see ''Discussion''; data not shown). NAC, N-acetylcysteine.

injury of the femur. We found that exposure to alcohol before blunt orthopaedic traumatic injury was associated with impaired fracture healing for at least 2 weeks after injury. In addition, we found that administration of N-acetylcysteine during the early postinjury period attenuated alcohol's effects on fracture callus strength and histologic structure, including restoration of normal tissue composition and endochondral bone formation. These results strongly suggest that the deleterious effects of alcohol on fracture healing are related to alcohol-induced oxidative stress.

After a fracture, osteoprogenitor cells and undifferentiated mesenchymal cells located in the adjacent periosteum are recruited to the site of injury to start the process of callus formation through both intramembranous and endochondral ossification mechanisms with endochondral bone formation occurring in the overlying external callus that originates from the fracture hematoma and recruited mesenchymal cells.[25] In our study, we observed immature chondroid-like tissue, fibrous tissue, and a notable absence of hypertrophic chondrocytes in external callus from binge alcohol-treated rats, indicating that alcohol consumption inhibits normal cartilage formation and subsequent endochondral bone formation during fracture repair. Cartilaginous callus formation at the site of injury and associated endochondral ossification were restored to near-normal levels in animals treated with NAC after injury. These data suggest that exogenous augmentation of

antioxidant levels during fracture callus formation is protective against alcohol-related defects in callus integrity.

Although the mechanisms of alcohol's effects on fracture healing potential remain incompletely understood, recent work has identified an important role for the canonical Wnt signaling pathway in normal fracture repair[26] and mesenchymal cell lineage commitment.[15,27] Our laboratory has demonstrated a connection between alcohol exposure and perturbation of canonical Wnt signaling activity both in intact bone[15] and in fracture callus[28] suggesting that alcohol-deficient fracture healing occurs, in part, through a targeted deregulation of canonical Wnt signaling. One possible connection between alcohol exposure and deactivation of the canonical Wnt pathway may be related to increased oxidative stress signaling activity.[16] The Forkhead Box O (FoxO) family of proteins are activated by increased oxygen radical activity and the Wnt signaling protein beta-catenin is an important coactivator of FoxO. Oxidative stress promotes FoxO binding to beta-catenin, which leads to FoxO transcription activation.[29] Beta-catenin is also an important coactivator in the Wnt pathway, and oxidative stress has been demonstrated to inhibit the Wnt pathway by shifting beta-catenin activity toward FoxO and away from binding to canonical Wnt transcription factor TCF.[17,18]

Based on these previous data, we hypothesized that binge alcohol exposure before injury would inhibit bone fracture repair and that antioxidant therapy would mitigate this effect. Our results support this hypothesis. Although the current study does not explore molecular mechanisms underlying the inhibition of callus formation caused by alcohol or the reversal of this effect by NAC, the data do support the supposition that these results could be the result of an alcohol-related increase in oxidative stress, which in turn inhibits canonical Wnt activity crucial for normal fracture repair. This possibility is supported by our histology data. The dramatic decrease in endochondral ossification activity observed in callus from alcohol-treated animals at 2 weeks postinjury is suggestive of a deregulation of canonical Wnt signaling activity. Previous studies have demonstrated that perturbation of the precise regulation of canonical Wnt signaling activity blocks chondrocyte hypertrophy and ensuing endochondral ossification activity in the growth plate[30] and that activation of beta-catenin-TCF-mediated transcriptional activity induces de novo endochondral bone formation.[6] Thus, a deactivation of canonical Wnt activity in the callus produces a callus histologic phenotype similar to what we observe in alcohol-treated rats. When our alcohol-treated rats were given NAC, we observed a qualitative normalization of callus histologic structure, including recovery of hypertrophic chondrocytes and endochondral bone formation activity to levels indistinguishable from normal fracture healing observed in saline control rats, suggesting that antioxidant treatment reduces oxidative stress and restores the normal balance of oxidative stress and canonical Wnt signaling activity in the fracture callus of alcohol-treated animals.

We found no statistically significant differences in fracture callus strength between alcohol- and saline-treated animals at 4 or more weeks postinjury. Because animals were treated with binge alcohol only before and not after bone fracture injury, the effects of alcohol on the repair process in our model, although significant, could be transient. It is possible that the downstream effects of binge alcohol-induced oxidative stress dissipate with time. In addition, administration of N-acetylcysteine was discontinued after 2 weeks, so the withdrawal of NAC may have allowed the alcohol-treated groups (with NAC and without NAC) to normalize relative to each other once the antioxidant effect was removed. Although the effects of alcohol on callus strength does diminish over time, effects on callus histologic structure were still readily apparent at later time points, suggesting that preinjury exposure to alcohol has long-lasting repercussions on the skeletal response to injury related to callus composition and structure. The replacement of cartilaginous tissue with fibrous repair tissue and/or increased intramembranous ossification activity is observed in nonstabilized fractures.[31] These alternate healing mechanisms could result in a partial augmentation of callus bridging strength in the absence of normal callus architecture in alcohol-treated animals. How (or if) the fibrous callus produced in alcohol-exposed animals is eventually replaced by a normal healed bone structure in our model is not known and would require a longer-term examination of callus structure during the remodeling stage of healing. Prolonged fibrous tissue formation in the callus is associated with delayed fracture healing.[32] Thus, the long-term effects of preinjury binge alcohol exposure on callus-remodeling activity and the eventual resolution of a bone fracture injury remain undetermined.

Limitations of the current study include use of a rodent model of alcohol exposure and traumatic orthopaedic injury; however, this model has been used in previous studies of traumatic orthopaedic injury.[24,33,34] Because a blunt trauma device was used to create the injury, it was impossible to ensure exact reproducibility in fracture site and orientation; some fractures were slightly oblique or more distal than anticipated. Although somewhat variable, fracture injuries sustained likely replicate the clinical presentations of traumatic closed femur fractures. NAC administration was continued for only 2 weeks because we could find no reports in the literature of administration of NAC beyond 2 weeks in a rat model. In retrospect, it might have been beneficial to carry out NAC administration for the full 4 or 6 weeks to determine how long the effect lasts.

In summary, this study provides evidence that exposure to alcohol before traumatic bone injury has detrimental effects on fracture repair and that administration of NAC was associated with a reversal of alcohol-related effects on callus strength and fracture callus histologic structure. Future studies may help delineate the mechanisms behind this effect. Further studies should also examine the long-term effects of NAC administration after alcohol exposure and traumatic injury to determine if NAC may indeed be a safe and useful agent in cases of orthopaedic injury complicated by alcohol intoxication.

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