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Impact of Alcohol on Bone Health, Homeostasis and Fracture repair

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Abstract

Purpose of review: Alcohol use continues to rise globally. We review the current literature on the effect of alcohol on bone health, homeostasis and fracture repair to highlight what has been learned in people and animal models of alcohol consumption.

Recent findings: Recently, forkhead box O (FoxO) has been found to be upregulated and activated in mesenchymal stem cells (MSC) exposed to alcohol. FoxO has also been found to modulate Wnt/ β -catenin signaling, which is necessary for MSC differentiation. Recent evidence suggests alcohol activates FoxO signaling, which may be dysregulating Wnt/ β -catenin signaling in MSCs cultured in alcohol.

Summary: This review highlights the negative health effects learned from people and chronic and episodic binge alcohol consumption animal models. Studies using chronic alcohol exposure or alcohol exposure then bone fracture repair model have explored several different cellular and molecular signaling pathways important for bone homeostasis and fracture repair, and offer potential for future experiments to explore additional signaling pathways that may be dysregulated by alcohol exposure.

Keywords

alcohol; fracture; mesenchymal stem cells; Wnt/ β -catenin signaling; oxidative stress; FoxO

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Introduction

Despite the knowledge and understanding behind alcohol consumption and its negative health effects, alcohol consumption continues to increase globally(1*). In fact, alcohol consumption is the number one substance use disorder, with one study showing that the total volume of alcohol consumed on average increased 70% from 1990 to 2017(1*). Alcohol consumption is known to affect many different organs and body systems. One organ system that is particularly affected by alcohol consumption is the skeletal system. The effects of alcohol on the skeletal system occur through both direct and indirect mechanisms, with the consensus that the overall effect of alcohol consumption is a negative effect on bone health and homeostasis. Therefore, the health effects of alcohol abuse on bone health are still an area for continuing research.

There are many alcohol exposure regimes and animal models employed to study the effect of alcohol on bone health and homeostasis. One model system that provides valuable insight into the complex mechanism involved in primary bone formation, remodeling and homeostasis is bone fracture repair. Bone fracture repair recapitulates early bone formation and offers a unique system to assess many aspects of bone growth, development and homeostasis in a short time window. When considering that alcohol consumption and incidences of traumatic injury, specifically orthopaedic injuries, are highly correlated, studying the effects of alcohol on bone fracture repair is one valuable tool to uncovering the negative health consequences on bone health and homeostasis. This review focuses on the negative health effects of alcohol on bone health and homeostasis by analyzing different models of alcohol consumption, but in large part focuses on what has been learned from bone fracture repair models.

Alcohol and bone

Osteoporosis is a major health problem worldwide that continues to increase in prevalence every year. The World Health Organization estimates the number of osteopenia and osteoporosis patients to be greater than 200 million people worldwide and is only second to cardiovascular disease as a global healthcare problem(2). Many risk factors contribute to the etiology of osteoporosis, however, excessive alcohol use is one established risk factor that continues to rise worldwide. While there is a debate whether a health benefit exists for light-to-moderate alcohol consumption, research examining the effects of alcohol on bone generally arrive on the consensus that heavy or chronic alcohol use is detrimental to overall bone health and homeostasis.

Chronic alcohol consumption has been described to affect multiple organs, body systems and molecular pathways. One mechanism by which chronic alcohol use resulted in skeletal abnormalities was noted in rats, where two research groups found that chronic alcohol disturbed vitamin D metabolism(3, 4). Turner, *et al.* showed that rats receiving 38% of their overall caloric intake from alcohol over a 10-month period showed reduced serum magnesium levels, increased 25-hydroxyvitamin D3, and decreased 1,25-dihydroxyvitamin D3. Alcohol consumption also led to reduced overall tibial lengths with increased medullary area and reduced trabecular bone. Another study by Mercer *et al.* found that vitamin D

supplementation protected against chronic alcohol-induced bone loss(5). Interestingly, the effect of alcohol on bone loss was related to the amount of drinking in this study. These researchers found that rodents receiving 10% of their total calories from alcohol had no effect on any of the measured bone parameters, whereas rodents that received 36% of calories from alcohol saw marked reductions in bone mineral density and bone strength. Combined, these studies suggest an alcohol-related increase in bone resorption that was not compensated by an increase in bone formation. Indeed, these findings are supported by several studies that show alcohol consumption increases osteoclastogenesis and osteoclast activity in rodents and humans(5–12). The disruption in bone homeostasis could account for bone loss by shifting the balance towards bone resorption. One mechanism that has been examined in rodents found that alcohol directly modulates gene expression in osteoblasts to induce expression of receptor activator of nuclear factor- κ B ligand (RANKL), resulting in increased osteoclastogenesis(13). Chen *et al.* found chronic alcohol exposed rodents had decreased tibial trabecular bone and bone mineral density, but elevated RANKL within the bone marrow. In the same study, RANKL mRNA expression was also found to be elevated in primary osteoblasts co-cultured in alcohol. Interestingly, the effect of alcohol on RANKL mRNA expression is restored to control levels when primary osteoblasts or rodents are treated with 17 β -estradiol. These findings suggest that estrogen can antagonize the effect of alcohol on bone homeostasis. One additional study found that binge alcohol decreased trabecular bone mineral density and compression strength in vertebral bone, while concurrently increasing RANKL expression in adult animals, supporting the hypothesis that alcohol exposure exacerbates bone loss through increased bone resorption(6) While evidence in rodents and humans support the finding that chronic alcohol exposure leads to increased resorption, one study in alcohol-consuming Rhesus Macaques found a decrease in bone resorption and bone remodeling(14). Gaddani *et al.* found that serum levels of bone resorption biomarker carboxy-terminal collagen crosslinks (CTX) were reduced in chronic binge alcohol exposed Rhesus Macaques compared to control Rhesus Macaques. In addition, cortical bone porosity and osteon density were decreased in chronic alcohol exposed animals compared to control animals. The differences between these findings and findings from rodents and human studies could be related to drinking pattern or blood alcohol concentrations and levels of consumptions. In fact, light to moderate alcohol consumption in humans shows a similar effect on reduced bone remodeling(15). However, these findings highlight an important limitation to using rodent systems; the apparent lack of Haversian remodeling of cortical bone in small rodents(16–18). However, bone remodeling is not completely absent in rodents; Woven and lamellar bone created during bone fracture repair are remodeled into mature cortical bone, making a fracture repair model an option to study the effect of alcohol on bone remodeling activity in rodents.

Alcohol-induced osteoporosis through increased osteoclast number and activity is not the only mechanism described in the literature. It is well documented that alcohol has a direct effect on bone anabolism as well. When compared to non-drinkers, chronic alcohol users have increased incidence of osteopenia accompanied with decreased serum bone Gla-protein (more commonly known as osteocalcin)(19, 20). Osteocalcin functions to bind and concentrate calcium within bone tissue. Osteocalcin is secreted solely by osteoblasts and is considered a biomarker for active bone formation; Levels of osteocalcin within patient

serum has been shown to positively correlate with increased bone volume and bone formation rate(21). A recent study showed ethanol consumption biomarker phosphatidylethanol (PEth) levels correlated with decreased osteocalcin in a study that included human immunodeficiency virus positive individuals(22). These findings suggest chronic alcohol users have decreased bone volume and bone formation rates. Interestingly, when chronic alcohol users abstained for 7 days they saw an increase in osteocalcin, suggesting the effect of alcohol use on serum osteocalcin is reversible and that inhibition of osteoblastic activity is one of the main mechanisms responsible for bone loss. These findings were later confirmed in another study that followed patients for 6 months. In this cohort, patients who continued to misuse alcohol showed a worsening of serum osteocalcin and increased osteopenia, whereas patients who abstained had improvements in both parameters(23). Recent evidence suggests alcohol inhibits expression of other integral bone structural components. Pedersen *et al.* reported the effect of chronic alcohol exposure on several bone structural and matrix proteins(24). In this study rodents were fed on an alcohol diet for 3 months before researchers performed RNA-Seq on mRNA isolated from the femur. Researchers found that all six bone collagens and matrix proteins osteonectin, sialoprotein, and decorin expression were decreased with alcohol exposure. The findings suggest alcohol may have a direct effect on osteoblasts.

When examining the direct effect of alcohol on osteoblasts, several studies found that even moderate quantities of alcohol inhibited *in vitro* osteoblast proliferation and induced cellular senescence(25–29). One study found alcohol activated p21 and p53 in rodent osteoblasts, leading to activation of senescence-associated β -galactosidase(29). These findings suggest that alcohol consumption may contribute to loss of bone mineral density by inhibiting osteoblast proliferation, while cellular senescence suggests a loss of osteoblast activity. Indeed, two human studies found that chronic alcohol users with reduced bone formation showed signs of inhibited osteoblastic activity without differences in resorption when compared to non-alcohol users(20, 30). Similar findings were also seen in rodents exposed to alcohol(31). Fanti *et al.* showed that the effect of alcohol on bone formation rate was dose dependent and the increasing alcohol dose concentration did not affect observed osteoclast numbers. These findings were supported by Chavassieux *et al.*, who reported that primary osteoblasts cultured *ex vivo* in alcohol had inhibited alkaline phosphatase activity(28), one osteoblastic enzyme necessary for mineralization and a marker of osteoblast activity.

While most studies focus on the chronic mode of alcohol consumption and acute exposure of cell cultures, there is evidence that binge or episodic excessive alcohol consumption leads to similar effects. Using a rodent model of binge alcohol, one group found tibial bone mineral density was reduced by 25% and accompanied with decreased serum osteocalcin, and that 30 days of alcohol abstinence reversed these pathologies(32). Interestingly, the loss of vertebral trabecular bone related to binge alcohol exposure was not reversed following a 30-day abstinence, suggesting binge alcohol has both short- and long-term consequences for bone homeostasis. Binge alcohol has also been shown to exacerbate bone loss in ovariectomized rodents in a model of post-menopausal osteoporosis(33). In these experiments, the researchers found that animals who received a binge alcohol paradigm following ovariectomy had more severe trabecular and cortical bone loss than the individual conditions. Interestingly, Kidder and Turner found that both a chronic low and high dose

alcohol diet did not enhance bone loss in ovariectomized animals(34), suggesting that binge versus chronic alcohol consumption may have differential effects on bone loss in ovariectomized rodents.

Several potential treatment modalities have been described in the literature to prevent the loss of bone associated with binge alcohol drinking. Intermittent parathyroid hormone has been shown to modulate bone homeostasis through bone formation and resorption(35, 36), and importantly, acute and chronic alcohol consumption has been shown to reduce serum parathyroid levels in pregnant, non-pregnant rodents, and humans(37–40). To this end, Callaci *et al.* found that intermittent parathyroid hormone mitigated the effect of binge alcohol on bone loss following ovariectomy(33). Another adjuvant therapy described focused on restoring vitamin D deficiencies that are common among alcohol abusers. As mentioned earlier, chronic alcohol use has been shown to reduce systemic vitamin D in both human and animal models. Using a binge alcohol paradigm, Wezeman *et al.* found that exogenous vitamin D prevented alcohol-induced tibial and vertebral bone loss(41). One family of adjuvants used frequently to prevent bone loss in osteoporotic patients are bisphosphonates. Several studies show that bisphosphonates can mitigate the effects of binge and chronic alcohol consumption in animal models, likely by mitigating the effect of alcohol on bone resorption(11, 42). Taken together, these findings suggest that the main mechanism of alcohol-induced bone loss is primarily through perturbing bone homeostasis resulting in a decrease in bone density over time.

Alcohol and fracture

Alcohol use is associated with an increased risk of bone fracture(43–46). In addition to bone fragility-related fractures(47), alcohol use is also associated with an increased risk of traumatic injury occurrence(48, 49). Indeed, nearly 50% of orthopaedic trauma patients have elevated blood alcohol at the time of injury(50, 51). The method of injury is also significant when comparing alcohol abusers and non-abusers. Alcohol abusers sustained twice as many fractures compared to alcohol non-abusers when falling at ground level and 3 times as many fractures when falling from higher levels(52). Alcohol abusers are also at a higher risk for developing fracture nonunion(53). Bone fractures can progress to nonunion, when the bone fails to develop an ossified fracture callus that completely bridges both ends of the fracture site. Long bones have a greater propensity of developing a fracture nonunion than fractures at other skeletal sites (54, 55*). The prevalence of fracture nonunion in long bones is estimated to be 5–10% of cases, requiring additional surgeries, prolonged patient recovery time and substantial increase in healthcare costs and morbidity(55*,56–58). One skeletal long bone in particular, the tibia, is prone to developing fracture nonunion, with nonunion rates as high as 19%(59). One factor that may be contributing to the development of a fracture nonunion in patients who abuse alcohol is bone fracture healing time. It has been shown that individuals who abuse alcohol also experience significantly longer bone fracture healing times compared to non-abusers(52). When comparing a population of adult male patients who sustained a tibial shaft fracture, those who abused alcohol took an average of 42 days longer for their fractures to heal. Interestingly, the difference in fracture healing time was only observed for a certain pattern of fracture sustained. There were no differences between alcohol abusers and non-abusers for oblique tibial fractures, but male patients who

abused alcohol had a longer healing time for transverse tibial fractures when compared to non-abusers.

In line with observations in human alcohol abusers, chronic alcohol consumption models in rodents also demonstrate evidence of inhibition of fracture healing. Chakkalakal *et al.* found that animals receiving 36% of their calories from alcohol for 6 weeks before and 6 weeks after a surgically administered fibula fracture had reduced fracture callus bone strength, stiffness and rigidity compared to pair-fed animals(60, 61). Interestingly, Chakkalakal *et al.* found that animals receiving alcohol before fracture and abstinence after injury had normal fracture callus biomechanical properties. Similar observations were found in another study investigating tibia fractures, but this study also found that even uninjured tibias also exhibited reduced stiffness and strength compared to pair-fed controls(62). Both studies found that chronic alcohol consumption led to reduced bone mass in the fracture when compared to pair-fed animals. These findings were supported by histological and radiographic evidence in a study by Elmali *et al.*, who found that animals who received alcohol had lower fracture healing scores and bone mineral density, respectively(63).

In rodents, binge alcohol exposure also significantly delays fracture healing and reduces biomechanical strength of the fracture callus(63–66). Upon histological examination, several studies show that alcohol significantly inhibits normal fracture callus formation(63, 66). In one study, Lauing *et al.* showed that alcohol-treated animals had reduced fracture callus size, reduced cartilaginous area within the fracture callus, and displayed decreased fracture callus biomechanical strength(66). Histological analysis in similar experiments by Roper, *et al.* found that animals receiving alcohol had significantly reduced fracture callus cartilaginous callus area and significantly reduced fracture callus hypertrophic chondrocyte area(67). These findings suggest that alcohol is likely detrimental to mesenchymal stem cell activity during homeostasis and at the site of fracture repair.

Effects of alcohol on mesenchymal stem cell activity and differentiation

Mesenchymal stem cells (MSCs) are multipotent cells that give rise to osteoblasts (bone producing cell) and chondrocytes(cartilage producing cell) lineages, which are necessary for fracture callus formation and repair. MSCs migrate from local periosteal tissue to the fracture site to initiate fracture healing(68–70). It has been reported that fracture callus tissue produces stromal cell-derived factor-1 and osteopontin to recruit MSCs to the fracture site following injury (71, 72) A recent study showed that rodents exposed to alcohol have reduced osteopontin expression within the fracture callus(73). In the same study, MSCs cultured *ex vivo* in alcohol were found to have reduced osteopontin-mediated migration and reduced integrin β 1 receptor expression, one receptor reported to play a role in MSC osteopontin-mediated migration(74, 75). CD44 protein expression, another known osteopontin receptor, was unaffected by alcohol exposure, suggesting MSCs utilize integrin β 1 receptor for osteopontin-mediated migration. These results suggest that one effect of alcohol on MSCs may be the inhibition of MSC recruitment to the site of fracture injury. Relatedly, Obermeyer *et al.* showed that intravenous administered MSCs were capable of migrating to the fracture site(76). In this study, animals binged with alcohol had improved fracture callus biomechanics and normalized histological and microcomputed tomography

after receiving intravenous MSCs. These findings suggest that endogenous MSC activity may be negatively affected following binge alcohol exposure. This hypothesis is supported by another study that examined the effect of alcohol on *ex vivo* cultured MSCs and found that alcohol induced MSC cellular senescence(27). In this study, Chen *et al.* treated MSCs with several concentrations of alcohol and examined the osteogenic potential after culturing the MSCs for 21 days later. Alcohol exposed MSCs had reduced mineralization and osteogenic marker mRNA expression when compared to control MSCs.

As covered earlier, several studies have shown that alcohol inhibits osteoblast activity and proliferation. Relatedly, it has also been reported that alcohol not only inhibits MSC proliferation and expansion, but impairs multipotentiality(77). To examine the effect of alcohol on MSC expansion and proliferation, Huff *et al.* placed rodents on liquid diets containing 36% alcohol for several weeks and found that animals on the alcohol diet had reduced MSC doublings/day and increased doubling time of *ex vivo* cultured MSCs(77). When compared to control MSCs, alcohol exposed rodent MSCs had a lasting effect on proliferation over several passage and perturbed capacity of MSCs to differentiate to osteoblast and adipocyte lineages.

Chronic alcohol exposure of rodents and non-human primates is associated with reduced bone density and increased bone marrow adiposity(78–81). In support of these findings, Chen *et al.* showed alcohol exposure enhances adipogenesis(82). In this study researchers co-cultured a well described MSC-like cell line, C3H10T1/2, in alcohol and adipogenic media and found that alcohol significantly elevated peroxisome proliferator-activated receptor gamma (PPAR γ), triglycerides, and activating protein 2 (AP2, a fatty acid carrier protein). There is also growing evidence to suggest alcohol induces transdifferentiation of osteoblasts towards adipocytes. One research group found that human MSCs co-cultured with alcohol and osteogenic media showed upregulated expression of PPAR γ , master regulator of adipogenesis, and contained increased number of lipid droplets, suggesting alcohol skewed osteogenic differentiation towards adipogenesis(83). In a similar experiments, Chen *et al.* found that MSCs treated with alcohol and osteogenic media had a significant suppression of Wnt/ β -catenin signaling and increased PPAR γ compared to control MSCs(84). The MSCs treated with alcohol in these experiments also had reduced mineralization and osteogenesis markers compared to control MSCs. Along these lines, Huang *et al.* used small interfering RNAs to inhibit PPAR γ expression in MSCs treated with alcohol and found that knockdown of PPAR γ rescued mineralization and osteogenic marker expression(85). These findings suggest a reciprocal relationship between Wnt/ β -catenin signaling and PPAR γ , redirecting MSC differentiation from osteogenesis towards adipogenesis. Taken together, these experiments suggest that alcohol's effect on cellular lineage commitment are due to dysregulated cellular signaling pathways that regulate MSC differentiation potential.

Effects of alcohol on signaling during bone homeostasis and fracture repair

Bone development, homeostasis and fracture repair are all regulated in part through Canonical Wnt signaling(86). Wnt receptor activation leads to stabilization of β -catenin and translocation into the nucleus where β -catenin promotes transcription of genes involved in bone formation and regeneration. Studies show that animals exposed to alcohol have dysregulated β -catenin within the fracture callus(66, 87). In one study, Lauing *et al.* showed that the downstream effector of the Canonical Wnt signaling pathway, β -catenin, was hyperphosphorylated in the fracture callus of rodents exposed to alcohol, targeting β -catenin to the proteasome for degradation(87). Wnt/ β -catenin signaling is tightly regulated during fracture callus formation. It has been reported that alterations in β -catenin levels or changes in post-translational modification can have positive or negative effects on fracture healing(88). One such protein that modifies β -catenin post-translationally is glycogen synthase kinase 3 beta (GSK-3 β). In a model of binge alcohol exposure, it was found that rodents had higher levels of active GSK-3 β (phospho-Y216) in the fracture callus of rodents who received alcohol compared to controls(87). Active GSK-3 β phosphorylates β -catenin, which destabilizing β -catenin and designates β -catenin for degradation(89). To this end, Lauing *et al.* showed exogenous activation of the Wnt/ β -catenin signaling pathway using GSK-3 β inhibitor lithium chloride post-fracture mitigated the negative effect of alcohol on fracture callus size and histological score(66). A recent study by Chen *et al.* showed MSCs and bone tissue from animals exposed to alcohol had increased expression and active levels of phosphatase and tensin homolog (PTEN)(90). Active PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) back to phosphatidylinositol 4,5-bisphosphate (PIP2), which is important for recruitment of phosphoinositide-dependent kinase-1 (PDK1) and subsequent phosphorylation and activation of AKT(91). Chen *et al.* found that alcohol-induced activated PTEN blocks AKT activation, which is necessary for phosphorylation and inhibition of GSK-3 β . PTEN-specific inhibitors restored osteogenic marker expression and improved rodent bone parameters when co-administered with alcohol, suggesting a possible therapeutic approach in the prevention of alcohol-induced osteopenia. Two additional studies describe alternative mechanisms in which alcohol may contribute to alterations in bone homeostasis through inhibition of Wnt/ β -catenin signaling. González-Reimers *et al.* found serum sclerostin, an inhibitor of Wnt/ β -catenin signaling secreted by osteocytes that inhibits osteoblast function, differentiation and survival, was significantly higher in alcoholic patients when compared to control patients(92). Alcoholic patients in this study also had decreased markers of bone formation and increased markers for bone resorption. The second study by Chen *et al.* found femurs from chronically alcohol-fed rodents had increased Wnt signaling antagonist dickkopf-related protein 1 (DKK1) when compared to control animals(82). Co-administration of an antioxidant attenuated the effects of alcohol on bone mineral density and osteogenic markers, and restored DKK1 to control levels in animals treated with alcohol.

The phosphatidylinositide 3-kinase (PI3K)-AKT-Mammalian (mechanistic) target of rapamycin (mTOR) signaling pathway has been postulated to be involved in bone homeostasis and some evidence suggests PI3K-AKT-mTOR signaling pathway may be

involved in MSC lineage differentiation(93). To this end, Liu *et al.* performed experiments to determine the effect of alcohol on PI3K-AKT-mTOR signaling in MSCs undergoing *ex vivo* osteogenic differentiation(94). In this study, researchers recapitulated previous findings that showed chronic alcohol exposure skewed differentiating MSCs cultured in osteogenic media away from osteogenesis and towards adipogenesis. Liu *et al.* reported that MSCs cultured in high dose alcohol and MSCs isolated from animals exposed to chronic high dose alcohol had activated PI3K-AKT-mTOR signaling and downregulated Runt-related transcription factor 2 (RUNX2, key transcription factor in osteoblast differentiation) and upregulated of PPAR γ . Co-administration of rapamycin, a mTOR-specific inhibitor, with chronic high dose alcohol prevented the upregulation of PPAR γ and rescued RUNX2 expression and MSC osteogenic differentiation, resulting in an attenuation of alcohol-induced bone loss and increased bone marrow adiposity. Similar findings were observed in a rodent model of alcohol-induced osteonecrosis(95), where Yang *et al.* and colleagues found that mTOR modulation rescued osteogenic marker expression and protected animals from alcohol-induced osteonecrosis.

Alcohol use leads to increases in systemic and intracellular reactive oxidative stress (ROS) (96). Furthermore, researchers have shown that MSCs and osteoblasts treated with alcohol directly produce ROS(27, 97, 98). To this end, several groups have reported on the effect of antioxidant treatments, e.g. N-acetylcysteine (NAC), in models of chronic alcohol exposure and bone fracture experiments combined with alcohol exposure(65, 67, 81, 82, 97, 99, 100). In a series of *in vitro* experiments in 2008, Chen *et al.* found NAC co-administered with alcohol blocked alcohol-induced osteoclastogenesis and RANKL expression in osteoblasts(97). As previously reported by Chen *et al.* in 2006, extracellular signal-regulated kinase (ERK) and downstream signal transducers and activators of transcription 3 (STAT3) were activated in response to alcohol exposure(13), but were attenuated when co-administered with NAC(97). These findings were mirrored in chronic alcohol experiments, where Chen *et al.* in 2010 showed NAC co-administered with alcohol prevented alcohol-mediated increases in osteoclast numbers, decreases in osteoblast numbers, and bone mineral density(100). Lastly, NAC co-administration also ameliorated senescence-associated p21 expression in the femurs of alcohol exposed animals(80). These findings suggest alcohol-induced ROS plays a major role in regulating bone homeostasis. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzymes have been shown to be upregulated in osteoblasts from animals exposed to alcohol, of which the primary subtypes expressed in response to alcohol were NOX1, NOX2, and NOX4, and alcohol-induced NOX1/2/4 upregulation is reversed when co-administered with NAC(97, 100). NOX enzymes are membrane-bound proteins that produce superoxides, which can be further converted in ROS. Interestingly, alcohol co-administration with NAC or a NOX-specific inhibitor prevents alcohol-induced ERK/STAT3 activation in osteoblasts, RANKL expression, and bone loss(97, 100). Experiments involving the use of animals with a loss-of-function (LOF) of NOX2 showed that NOX2 activity is necessary for alcohol-induced bone loss(9). NOX2 LOF animals exposed to alcohol were protected from alcohol-induced increases in serum CTx, suggesting NOX2 function is necessary for alcohol-induced bone resorption. However, NOX2 LOF animals saw no protection from alcohol-induced decreases in serum alkaline phosphatase and osteocalcin, suggesting that alcohol inhibition of

osteoblasts occurs independently of functional NOX2(9). In the same study, Mercer *et al.* treated an osteoblast cell line with a NOX2-specific inhibitor and found that NOX2 was solely responsible for the production of ROS after alcohol exposure and increased RANKL expression. Recent studies investigating whether knockout of whole-body NOX4(101) or MSC-specific NOX4(24) were protected against the deleterious effects of alcohol on bone formation revealed the effect of alcohol on osteoblast functioned independently of NOX4. While NOX4 knockout animals had increased cortical thickness and density, further studies are needed to determine the role of NOX enzymes in alcohol-induced bone loss. Taken together, these findings illustrate one alcohol-mediated signaling cascade where alcohol exposure upregulates NOX proteins, leading to ROS generation that activates ERK/STAT3 signaling and subsequent RANKL expression. Similar effects have been observed in bone fracture models. One study performed by Duryee *et al.* found NAC reduced tissue levels of oxidative stress, RANKL expression, and osteoclast activity while improving fracture callus biomechanical strength(99). In a study of binge alcohol exposure, rodents treated with NAC following an alcohol binge had an improvement in fracture callus size, biomechanical properties and histological appearance compared to animals receiving alcohol binge alone(67). Combined, these findings suggest the physiological response to alcohol and alcohol metabolism have profound effects on bone health and homeostasis that may benefit from antioxidant therapies.

There are several cellular mechanisms capable of responding to oxidative stress. One intracellular molecular responder is the Forkhead box O (FoxO) family of transcription factors. In a study in 2016, Roper *et al.* found that the fracture callus of alcohol exposed rodents had elevated levels of total and active FoxO1 phosphorylated at S207 concurrent with reduced levels of inactive FoxO phosphorylated at S253(67). In the same study, the authors found that the FoxO signaling perturbed by alcohol exposure was restored to levels of saline treated rodents by NAC treatment following fracture(67). The fracture callus is produced by the differentiation of MSCs into osteoblasts and chondrocytes, and evidence from fracture studies suggest that alcohol is disrupting cellular processes in early fracture callus development(67). To this end, a recent study by Sharieh *et al.* examined the effect of alcohol on *ex vivo* MSC differentiation and FoxO1/3 signaling(102*). In this study, researchers found MSCs cultured in alcohol had increased FoxO1/3 expression and activity when compared to control MSCs. Past studies found MSCs cultured in alcohol had reduced multipotentiality(77). Similarly, Sharieh *et al.* found that primary MSCs co-cultured with alcohol and osteogenic or chondrogenic media had reduced marker expression for MSC osteochondral lineage differentiation(102*). The effect of alcohol on osteogenic and chondrogenic lineage marker expression was mitigated when FoxO1/3 expression was knocked down using both pharmacologic and genetic approaches in MSCs cultured in alcohol(102*). These findings suggest that the activation of FoxO signaling by alcohol impairs MSCs osteochondral differentiation. Canonical Wnt/ β -catenin signaling is necessary for the commitment of MSCs into osteoblasts and chondrocyte cell lineages, which initiate repair and produce the fracture callus(53–56). Further studies are necessary to determine whether the inhibition of cartilaginous fracture callus formation in animals exposed to alcohol is the result of inhibition of fracture site MSC differentiation (Figure 1). Interestingly, FoxO is known to modulate Wnt/ β -catenin signaling by sequestering β -catenin

from the Wnt signaling pathway(103*,104,105*). Almeida *et al.* found that an osteoblast cell line treated with an oxidative stressor, hydrogen peroxide, increased FoxO and β -catenin association, which increased expression of FoxO-mediated transcription and decreased osteoblast differentiation. In a separate study, Almeida *et al.* found that rodents lacking FoxO1/3/4 in osteoprogenitor cells have increased bone mass, bone formation, and increased Wnt signaling(106). In addition, osteoprogenitor-specific FoxO knockout animals had reduced bone marrow adiposity, suggesting osteoprogenitor cells were skewed away from adipogenesis towards osteogenesis.

Another important signaling pathway in bone growth and homeostasis is the transforming growth factor beta (TGF- β) superfamily, including the bone morphogenetic protein (BMP) signaling pathway. BMP-2 activity is not required for normal bone formation and homeostasis, but experiments using BMP-2-deficient animals found that BMP-2 is required for the initiation of fracture healing(107). In a study of binge alcohol exposure, Bratton *et al.* found that animals receiving chronic binge alcohol before sustaining a tibial fracture had increased BMP-2 and BMP receptor 2 within the fracture callus(108). BMP-2 is also known to bind and activate BMP receptor 1a, which is necessary for osteoblast extracellular matrix deposition(109). In the same study researchers found that BMP receptor 1a was found to be decreased in animals receiving alcohol when compared to control animals. BMP-2 signaling can be modulated by endogenous BMP-2 antagonist, chordin. A previous study by Kloen *et al.* found BMP-2 inhibitors like chordin are increased in fracture nonunion tissue(110). Bratton, *et al.* found that chordin levels were increased in the fracture callus of animals exposed to alcohol. Taken together, these data suggest alcohol exposure perturbs BMP-2 signaling within the fracture callus and may predispose a fracture towards nonunion. Another component of the TGF- β superfamily that is important for fracture repair is TGF- β 1(111). TGF- β 1 has been shown to function as a chemoattractant in MSC migration and to promote proliferation of osteoblasts and chondrocytes(112), which are necessary for proper fracture callus formation. In a study of binge alcohol exposure, Driver *et al.* found that animals exposed to alcohol had decreased levels of TGF- β 1 in fracture callus tissue(113). These findings offer further evidence that alcohol dysregulates TGF- β and BMP signaling in fracture callus of alcohol exposed animals.

In the early inflammatory phase of bone fracture repair, several cytokines and chemokines are generated from surrounding and injured tissue to attract immune and stem cells to initiate fracture repair as part of the normal physiological response. Tumor necrosis factor alpha (TNF α) and interleukin 1 beta (IL-1 β) are two pro-inflammatory cytokines that have been long shown to have negative effects on normal bone formation(114, 115). Indeed, TNF α and IL-1 β antagonism has been shown to improve bone formation(116). In 2003, Perrien *et al.* reported that chronic alcohol exposure in a bone fracture model led to elevated levels of TNF α and IL-1 β in fracture tissue(117). Interestingly, chronic alcohol exposed animals receiving TNF α and IL-1 β antagonists on the day of bone fracture had improved bone bridging and improvements in bone mineralization(118). Later experiments targeting only TNF α signaling revealed the TNF α was the major signaling axis in alcohol inhibition of a distraction osteogenesis fracture repair model. Wahl *et al.* utilized a systemically administered soluble TNF receptor 1 (TNFR1) derivative to adsorb excess TNF α generated in chronic alcohol exposed animals(119). Alcohol exposed animals receiving the soluble

TNFR1 derivative had improved distraction osteogenesis bridging and histological evidence of new bone formation as compared to control animals. Experiments utilizing transgenic TNFR1 knockout animals confirmed that the increased TNF α generated in alcohol exposed animals inhibited distraction osteogenesis through TNFR1(120). Similar findings were observed in experiments measuring the effect of alcohol on bone formation. In these experiments, Shankar *et al.* found soluble TNFR1 derivative abolished the negative effect of alcohol feeding on bone formation and reduced expression of adipogenic marker expression in bone tissue(81). Yu *et al.* found that one potential source of elevated TNF α generation was generated from MSCs(121). Cultured MSCs from alcohol-induced osteonecrosis patients expressed TNF α nearly 2-times that of healthy MSCs over a 21-day period. Alcohol-induced osteonecrosis patient MSCs also had reduced osteogenic potential, with decreased osteogenic marker expression when undergoing osteogenic differentiation as compared to control MSCs. In the same study, researchers treated healthy MSCs with exogenous TNF α and found that TNF α directly inhibited osteogenesis. Whereas healthy MSCs treated with a well-known TNF α inhibitor, Adalimumab, improved osteogenesis. Microarray analysis revealed miR-31 was upregulated in alcohol-induced osteonecrosis MSCs patients and TNF α -treated MSCs. Importantly, miR-31 mimic transfections inhibited two important osteogenic transcription factors, RUNX2 and special AT-rich sequence-binding protein 2 (SATB2). These findings suggest TNF α signaling works to inhibit osteogenesis by upregulation of miR-31, leading to downregulation of RUNX2 and SATB2.

Conclusion

Much work has been done to study the effect of alcohol on bone and bone fracture. The studies reviewed in this article illustrate the need for further research and suggest areas of research needed to fill gaps within the current literature. One aspect of this research that appears consistent is that alcohol's effect on bone remodeling, formation and fracture healing is likely mediated through the suppression of MSC-mediated bone formation activities and activation of osteoclast-mediated bone resorption.

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List of Abbreviations

AP2	Activating Protein 2
BMP	Bone Morphogenetic Protein
CTx	Carboxy-terminal collagen crosslinks
DKK1	Dickkopf-related protein 1
ERK	Extracellular signal-regulated kinase

FoxO	Forkhead box O
GSK-3β	Glycogen synthase kinase 3 beta
IL-1β	Interleukin 1 beta
LOF	Loss-of-functionm
TOR	Mammalian (mechanistic) target of rapamycin
MSC	Mesenchymal stem cell
MSCs	Mesenchymal stem cells
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH Oxidase
PI3K	Phosphatidylinositide 3-kinase
PEth	Phosphatidylethanol
PDK1	Phosphoinositide-dependent kinase-1
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PPARγ	Peroxisome proliferator-activated receptor gamma
PTEN	Phosphatase and tensin homolog
RANKL	Nuclear factor- κ B ligand
RUNX2	Runt-related transcription factor 2
ROS	Reactive Oxygen Species
SATB2	Special AT-rich sequence-binding protein 2
STAT3	Signal transducers and activators of transcription 3
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TNFR1	TNF receptor 1

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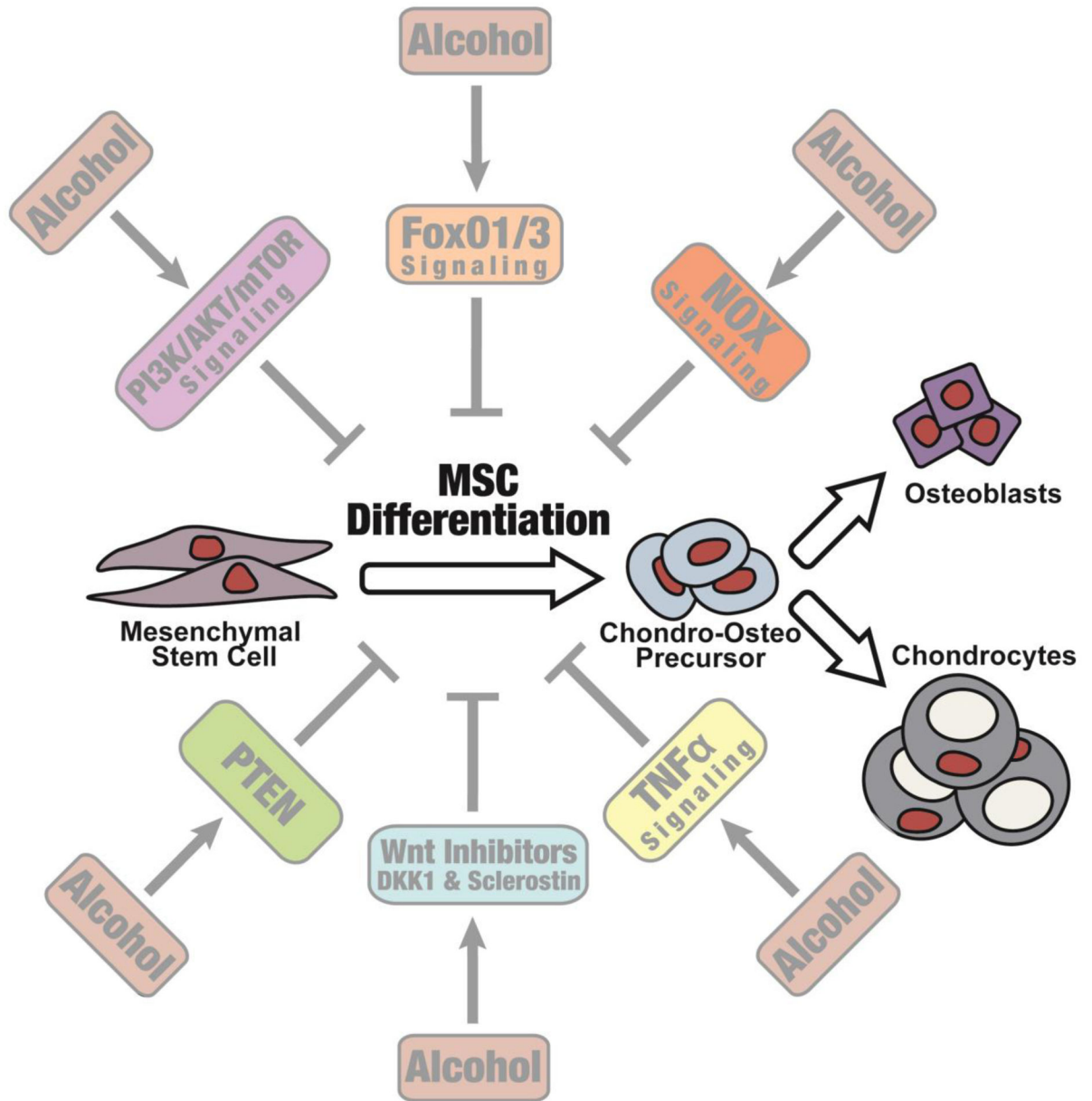


Figure 1:
Hypothesized effect of alcohol inhibition in mesenchymal stem cells at the fracture site.m