N-acetyl Cysteine (NAC) Restores Normal Onset of Osteogenesis in a Fracture Model of Chronic Ethanol-Fed Rats

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Introduction:
Chronic consumption of alcohol (“alcoholism”) compromises host defense to pathogens and to traumatic injury and prolongs recovery from injury. Fracture healing in alcoholics is delayed and is often associated with infections. In fractures in rat models of alcoholism, bone regeneration is inhibited; instead, a scar tissue dominated by immature cartilage and fibrous tissue is observed. Clearly there is a disruption of the normal biological process of fracture healing associated with alcoholism. The mechanisms of this failure have been investigated, but remain to be established. We hypothesize that this failure is initiated by excessive oxidative stress due to alcoholism that overrides the body’s antioxidant defenses and disrupts the early phase of fracture healing. Proinflammatory cytokines normally released in the fracture site have specific and time-sensitive roles in the onset of osteogenesis. But, abnormal expressions of these cytokines inhibit the process. The hypothesis of this study is that treatment with the antioxidant n-acetyl cysteine (NAC) reduces the oxidative stress and restores the normal onset of osteogenesis.

Methods:
The experimental protocol was approved by the IACUC at Omaha Veterans Affairs Medical Center. By comparing the human ages of reaching skeletal maturity and peak bone mass with the ages in the male Wistar rat when growth of long bones slows and reaches a plateau and when peak bone mass occurs [1], we estimated that 21 weeks of ethanol feeding of the 10-month old rat represents approximately 25 years of adult human alcoholism. A closed fracture was produced [2] in the right femur of male Wistar rats that were fed the Lieber DeCarli ethanol diet (LDE) or control diet (LDC) daily during 21 weeks starting at the age of 10 months. All rats were euthanized 8 days after fracture. Four groups of rats (n=5) were used. Group A (controls) fed LDC by pair feeding (PF) with LDE-fed rats in group B; both groups A and B were treated with saline by i.p. injection (control for NAC) on days 4 to 7 post fracture. Group C and D were the same as A and B, respectively, but with 200 mg/kg b.w. NAC treatment on days 4 to 7 post fracture. We performed assays listed below using blood serum and homogenized bone tissue from the fractured femur. Serum was assayed for malondialdehyde (MDA) in the thiobarbituric acid reacting substances (TBARS) assay, IL-1β, TNF-α, IL-6, IFN-γ, bone alkaline phosphatase (BALP), vitamin D3, band 5 tartrate-resistant acid phosphatase (TRAP5b), crosslinked C-telopeptide of type-I-collagen (CTX) and osteoprotegerin (OPG) using commercially available kits. The fractured femurs were collected, homogenized in PBS, the protein quantified and the bone homogenate assayed for TBARS, IL-1β, TNF-α, IL-6, IFN-γ, osteocalcin, TRAP5b, CTX, OPG and receptor activator factor of nuclear factor kB ligand
(RANKL) using commercially available kits. Statistical analysis by ANOVA and multiple comparison procedures (Student-Newman-Keuls Method) were used to determine differences.

Results:
Four of the 20 rats died prematurely leaving 4, 3, 5 and 4 rats in groups A, B, C and D, respectively, for the study. Oxidative Stress. There was a 2.7-fold increase in serum MDA in ethanol-fed rats (43.7 ± 1.3 μM, p = 0.002) compared with PF controls (16.3 ± 2.1 μM). NAC decreased this significantly (31.8 ± 2.4 μM, p < 0.05). In bone homogenate, the 4-fold increase (p = 0.013) due to ethanol (6.9 ± 3.1 μM) vs. control (1.7 ± 0.3 μM) was reduced by 80% by NAC to control level (1.4 ± 0.2 μM, p = 0.035).

Proinflammatory Cytokines (Table 1). Ethanol increased serum markers of TNF-α (13-fold, p < 0.001) and IL-6 (0 to 219 pg/ml, p = 0.018). NAC treatment of ethanol-fed rats decreased these elevated serum levels: TNF-α by 34% (p = 0.051) and IL-6 by 98% (p = 0.008). Fracture Healing (Table 1). Bone formation markers, BALP and osteocalcin, were not significantly different among the four groups. Serum level of Vitamin D3, one of the regulatory molecules in the early phase of fracture healing, was increased 84% (p = 0.007) by ethanol. NAC treatment decreased this by 34% (p = 0.01). In bone, ethanol increased the marker for osteoclast function TRAP5b 3.5-fold (p < 0.001) and NAC decreased it by 85% to control level (p < 0.001).

Table 1. Effects of Ethanol and NAC on Early Phase of Fracture Healing [p ≤ 0.05: Ethanol vs. Control (*); Ethanol + NAC vs. Ethanol (**)]

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>Vitamin D</th>
<th>TRAP5b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Serum</td>
<td>Serum</td>
<td>Bone</td>
</tr>
<tr>
<td>Control</td>
<td>2.5±2.5</td>
<td>0±0</td>
<td>30±6.6</td>
<td>2.9±0.8</td>
</tr>
<tr>
<td>Ethanol</td>
<td>32±3.2*</td>
<td>219±124*</td>
<td>54±3.4*</td>
<td>10±0.3*</td>
</tr>
<tr>
<td>Control + NAC</td>
<td>10±4.4</td>
<td>0±0</td>
<td>35±2.4</td>
<td>2.4±0.6</td>
</tr>
<tr>
<td>Ethanol + NAC</td>
<td>21±0.4**</td>
<td>4.5±4.5**</td>
<td>36±2.3**</td>
<td>1.6±0.7**</td>
</tr>
</tbody>
</table>

Discussion:
The TBARS/MDA data support our hypothesis that NAC treatment completely eliminates the excess oxidative stress in the fractured bone. Immunodeficiency induced by chronic, but not short-term, ethanol in human alcoholics and in mice is marked by T cell activation and increased cytoplasmic levels of IFN-γ [3]. Ethanol increased IFN-γ in serum and bone in the present study, which was mostly prevented by NAC treatment (data not shown). This is significant because in human alcoholics delay in fracture healing is often associated with infections occurring in the early phase of healing. NAC treatment also restored the elevated serum levels of cytokines to control levels completely (IL-6) or partially (TNF-α). TNF-α and IL-6 are critical for the inflammatory response that triggers osteogenesis. TNF-α exhibits a biphasic response, with high levels expressed immediately following injury that become undetectable within 72 hours [4]. Thus, the high serum levels of these cytokines persisting 8 days after fracture in ethanol-fed rats indicate that normal onset of osteogenesis did not occur. But NAC treatment of ethanol-fed rats
abolished this excess and hence would have restored the normal process. During 3 to 10 days after fracture in rat femur, serum vitamin D3 level decreases rapidly and is localized in the fracture callus, where it participates in the regulation of early phase of fracture healing [5]. Ethanol apparently prevented this localization to callus by increasing the serum level, which was reversed by NAC. Osteoclast activity in fracture callus is part of the coordinated bone formation-resorption activities for remodeling the newly formed bone into mature bone [4], but excessive resorption due to ethanol (increased TRAP5b in bone) would disrupt this process. But NAC decreases this to control levels restoring the normal remodeling. We conclude that in an experimental model of chronically ethanol-fed rats representing 25 years of human alcoholism, NAC treatment reduces excess oxidative stress, overcomes immunodeficiency and restores normal proinflammatory cytokine expressions, and thus prevents ethanol-induced disruption of the early phase of fracture healing.

**Significance:**
There is strong experimental evidence that delay or failure of fracture healing in chronic alcoholics, often associated with infections, results from disruption of the biology of fracture healing by alcohol, and not just due to noncompliance of patients. The present study provides evidence that alcohol induces excessive oxidative stress which disrupts the early phase of fracture healing and that normal healing may be restored by early short term treatment with the antioxidant n-acetyl cysteine.

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