CHRONIC ETHANOL CONSUMPTION RESULTS IN DEFICIENT BONE REPAIR IN RATS

DENNIS A. CHAKKALAKAL1,2,3*, JERZY R. NOVAK1,3, EDWARD D. FRITZ1,2, TERESA J. MOLLNER1,2, DANIEL L. MCVICKER1, DENISE L. LYBARGER1, MICHAEL H. MCGUIRE2 and TERRENCE M. DONOHUE, Jr1,3

1Omaha Veterans Affairs Medical Center, Omaha, NE 68105, 2Creighton University School of Medicine, Omaha, NE 68178 and 3University of Nebraska Medical Center, Omaha, NE 68198, USA

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Abstract — There is evidence that ethanol inhibits osteoblast function and that chronic ethanol consumption induces systemic bone loss and increases the risk of fracture in humans. The purpose of the present study was to determine whether chronic ethanol consumption also compromises the healing of injured bone. Male Sprague–Dawley rats, 8–10 weeks old, were placed into four feeding groups: group A received ethanol (36% of calories) as part of a liquid diet; group B was pair-fed to group A and received an isocaloric control diet containing maltodextrin; group C was fed the AIN-93M standard semi-purified liquid diet ad libitum; group D was fed the same ethanol diet as group A before bone injury, but after surgery (see below) these rats were given isocaloric control diet ad libitum. After 6 weeks on their respective diets, a bone repair model was surgically created at the midshaft in both fibulae of each rat. Seven weeks after injury the animals were euthanized and bone healing was evaluated by determining rigidity of the fibula by three-point bending, flexural modulus of the repair tissue and mineral content of the repair tissue. Rigidity of fibula in ethanol-fed rats and their pair-fed controls (groups A and B) were respectively 48 and 47% lower than in group C. Flexural modulus of the repair tissue in ethanol-fed rats had a 55% (P = 0.046) deficiency compared with their pair-fed controls. The mineral contents in groups A and B were respectively 16 and 13% lower than in group C. There were no significant differences in the results between groups C and D. Thus, the outcome of bone repair in ethanol-fed rats was deficient compared with rats receiving a standard maintenance diet. The repair tissue in ethanol-fed rats was mechanically inferior to that in pair-fed controls. This deficiency could not be attributed to the reduced food consumption of these animals. On the other hand, the restoration of normal bone healing in group D cannot be attributed solely to the cessation of ethanol feeding after bone injury because of the increased food consumption during this period.

INTRODUCTION

Clinical studies demonstrated that chronic alcohol consumption is associated with osteopenia and increased risk of fractures (Bikle, 1993; Klein, 1997; Nyquist et al., 1997b). In addition, the fracture healing process itself may be compromised by alcohol misuse, since fracture treatment using standard protocols encounters a significantly higher incidence of complications in alcohol misusers (Adell et al., 1987; Tennesen et al., 1991; Passeri et al., 1993; Nyquist et al., 1997a). It is not known whether these complications occur because of direct adverse effects of alcohol on fracture healing or indirect effects incidental to chronic alcohol consumption.

The mechanism of alcohol-associated osteopenia appears to involve a direct effect of alcohol on bone cells that leads to suppression of new bone formation, without any indirect or modulating role for mineral regulating hormones (Sampson, 1997). Alcohol has been shown to have an antiproliferative effect on osteoblastic cells in culture (Friday and Howard, 1991; Chavassieux et al., 1993) and in ethanol-fed rats (Dyer et al., 1998). It also inhibits osteoblast function in vitro (Friday and Howard, 1991; Chavassieux et al., 1993) and in vivo (Garcia-Sanchez et al., 1995; Diez et al., 1997; Dyer et al., 1998).

We chose to investigate the effect of alcohol on bone healing using rats given ethanol as 36% of total calories as part of a liquid diet, since this experimental model has been used in studies of alcohol-induced bone loss (Baran et al., 1980; Turner et al., 1987; Peng et al., 1988; Kusy et al., 1989; Turner et al., 1991; Hogan et al., 1997; Sampson et al., 1997, 1998). The liquid diet feeding model demonstrates steatosis and hepatomegaly, which are the early stages of alcoholic liver disease (Donohue et al., 1987). Bone loss occurs in both the cancellous and compact bone regions in the ethanol-fed rat (Baran et al., 1980; Peng et al., 1988; Hogan et al., 1997; Sampson et al., 1997, 1998), as it does in human alcoholics (Bikle et al., 1985; Diamond et al., 1989; Peris et al., 1992). Chronic ethanol feeding of rats causes deficiency in bone matrix synthesis and mineralization, resulting in inferior microstructural architecture and mechanical properties of bone (Baran et al., 1980; Turner et al., 1987; Peng et al., 1988; Kusy et al., 1989; Turner et al., 1991; Hogan et al., 1997; Sampson et al., 1997, 1998). These results suggested us that the repair process in a bone injury in the diaphysis of long bones in these rats may be adversely affected by a similar inhibition of new bone formation which would lead to a deficient bone healing outcome.

The fracture healing process in the long tubular bones of rodents differs from that in humans. In an effort to create a bone repair model in rat long bone that provides a better representation of the human bone healing process than conventional fracture models in the rat, we deliberately altered the bone repair process by surgically creating a segmental defect in rat fibula and fitting it with a tissue scaffold as previously described (Chakkalakal et al., 1999, 2001). We have demonstrated the sensitivity of this model to detect the effect of early biological deficiencies on the outcome of bone repair at 7 weeks after injury (Chakkalakal et al., 2000). The specific objectives of this study were to determine whether: (1) the outcome of bone repair in rats receiving ethanol as part of a nutritionally adequate liquid diet was deficient compared with pair-fed control rats and rats receiving a standard maintenance diet; (2) ethanol affects bone repair in addition to any effect of...
reduced food intake often associated with ethanol consumption; (3) stopping ethanol consumption after bone injury restores the normal outcome of bone repair or, at least, improves it.

MATERIALS AND METHODS

Animal maintenance

This research protocol was approved by the Animal Studies Subcommittee and the Research & Development Committee of the Omaha Veterans Affairs Medical Center. Thirty-six 8–10-week-old male Sprague–Dawley rats ranging in body weight from 237 to 298 g (Charles River Breeding Laboratories Inc., Wilmington, MA, USA) were separated into four groups of nine each and housed individually. Groups A and B were matched by body weight and pair-fed Lieber–DeCarli liquid diet (Dyets Inc., Bethlehem, PA, USA) (Lieber and DeCarli, 1982) containing either ethanol (36% of total calories; group A) or isocaloric maltodextrin (control diet; group B). The volume of food consumed by each ethanol-fed rat in group A was measured daily and this amount was given to its pair-fed control rat in group B the next day. The daily food consumption of each rat in group B was also recorded. A third group of rats (group C) was given the standard semi-purified liquid diet AIN-93M ad libitum. A fourth group (D) was given the AIN-93M liquid ethanol diet, but after surgery the animals were switched to the liquid control diet, fed ad libitum, for 7 weeks until death. All ethanol-fed animals were acclimated to their diet over a three-day period by giving ethanol as 12, 24 and 36% of total calories on the respective days. To prevent animal mortality due to rapid ethanol withdrawal in group D, 24 and 36% of total calories on the respective days. The dried extract was saponified at 60°C for 30 min in 90% ethanol containing 0.8 N KOH. Two volumes of 0.15 M MgSO4 were added to the mixtures, which were then centrifuged to precipitate fatty acids. The supernatant fractions were assayed for glycerol (derived from triacylglycerol), using the enzymatic assay kit purchased from Sigma Diagnostics (Cat. #302A) (St Louis, MO, USA). Blood-ethanol concentrations were determined by gas chromatography using the headspace technique (Eriksson et al., 1977).

Procedures for evaluation of bone repair

The right and left fibulae were excised from all animals immediately after collecting the livers and blood samples. The fibula was separated from the tibia by dissection at the synchondrosis (proximal) and synostosis (distal) with a scalpel and the tibia was discarded. The fibulae were cleaned of adhering soft tissues, and kept moist in normal saline in closed vials. In groups C and D, the outer surface of the DBM scaffold was mineralized and the soft tissue was easily separated without disturbing the bone repair site. In groups A and B, although the outer surface of DBM was less mineralized and hence softer, it was possible to separate the soft tissue from the bone repair tissue, without disturbing the latter, by careful dissection. The fibulae were used for various bone assays as described below.

Mechanical properties of the whole bone and the repair tissue

Three-point bend tests of both fibulae from each rat were performed using methods developed in our laboratory for canine bones (Chakkalakal et al., 1990) and later adapted to rat bones (Chakkalakal et al., 1996, 1999). Bend tests were performed on a materials testing machine (Instron Model 1011) within 40 min after the death of the rat, keeping the bones wet in 0.9% saline during this period. The bone was supported on roller supports 15 mm apart with the repair site centred within the test span. Loads of 10–200 g were applied, with the load-applicator moving at the rate of 1 mm/min. The load–deflection graph has a linear portion and the test was stopped before reaching the yield point. The vertical diameter ($D_v$) and the horizontal diameter ($D_h$) of the fibula at the site of load application were measured using precision calipers. Instron-supplied software was used to calculate rigidity, defined by (Dumbleton and Black, 1981; Torzilli et al., 1981):

$$ R = \frac{(\text{slope})L}{48} $$

(1)

where ‘slope’ is that of the linear portion of the load–deflection graph and $L$ (= 15 mm) is the test span. The shape of the cross-section of the bone at the repair site was
approximately elliptical. Therefore, the area of cross-section ($X_t$) and the area moment of inertia for the direction of loading in the bend test ($I$) were calculated as follows (Eshbach and Souders, 1975):

\[ X_t = \pi (D_v/2)(D_u/2) \]
\[ I = (\pi/64)D_u^2 D_v^3 \]

(2)
(3)

In addition to rigidity, which is a property of the whole bone, we evaluated intrinsic (i.e., material) properties of the repair tissue by determining the flexural modulus ($\mu$) derived from simple beam theory (Dumbleton and Black, 1981; Torzilli et al., 1981):

\[ \mu = R/I. \]

(4)

Mineral content of the repair tissue

The method used in our laboratory has been previously described (Chakkalakal et al., 1999). After the bend test, a 4-mm segment of the fibula consisting of the entire repair site including the DBM scaffold was excised. These samples were placed in crucibles, dried at 110°C for 24 h, weighed and then ashed at 700°C for 24 h. Ash weight was calculated as a percentage of dry tissue weight.

Statistical analysis

Data analysis was performed using descriptive statistics to determine mean and 95% confidence interval for each variable. To take into account the non-independent status of two samples (fibulae) from the same animal, overall analysis of variance for a one-factor nested design. Multiple pairwise comparisons corresponding to the specific objectives stated in the Introduction were made using the Holm–Shaffer sequential Bonferroni procedure to maintain experimentwise type I error at the $P \leq 0.05$ level (Shaffer, 1986). Comparisons that were not statistically significant are designated n.s.

RESULTS

Three animals in the ethanol-fed group (A) died during the first week after bone surgery. Their corresponding pair-fed control animals in group B were excluded from the data analysis.

In addition, one fibula sample was lost due to slippage of the DBM scaffold from the repair site from three of the groups: B (pair-fed), C (AIN-93M-fed) and D (switched from ethanol to control diet ad libitum after surgery). Thus the number of samples available for determination of mean rigidity in the four groups (A, B, C and D) were 12, 11, 17 and 17, respectively. After the bend test, two, one, one and two samples were selected randomly from groups A, B, C and D, respectively, for histology. The remaining 10, 10, 16 and 15 samples, respectively, were used for determination of mineral content of the repair tissue. The blood-ethanol concentration (mean ± 95% confidence interval) detected in the morning on the day of euthanasia of the ethanol-fed rats (group A) was 5.6 ± 4.7 mM. Euthanasia was performed in all animals at 7 weeks post-surgery to evaluate outcome of bone repair and changes in the liver.

Gain of body weight during the protocol

The body weights of the four groups of rats at three time points during the protocol (start, surgery and end) and the changes in body weight between these time points are given in Table 1. Rats in pair-fed groups A and B had nearly identical starting body weights due to weight-matching, but this was 12 and 17% lower than in groups C and D respectively. However, at the time of surgery, group D had almost the same average body weight as groups A and B. Only group C, which received the AIN-93M diet, had a larger gain, so that it was significantly higher than the other three groups at the time of surgery. The average rate of weight gain of rats in group D before surgery was 77% less than that of group A rats, in spite of nearly identical rates of consumption of the liquid ethanol diet (55 vs 52 ml/day/rat). During the 7-week post-surgery period, group D rats consumed the control diet at the rate of 89 ml/day/rat compared to 64 ml/day/rat in group A (and B). As a result, the average rate of weight gain of group D rats post-surgery was 2.6-fold that in group A. The overall rate of weight gain during 13 weeks of the protocol was the same for groups A and B, which was significantly lower than that for groups C and D.

Changes in the liver

Table 2 shows relative liver weights and protein contents after 13 weeks of feeding (and 7 weeks after bone surgery). These were the same in all four groups. Despite the absence

<table>
<thead>
<tr>
<th>Group</th>
<th>Starting weight (g)</th>
<th>Pre-surgery weight (g)</th>
<th>Final weight (g)</th>
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<tbody>
<tr>
<td>A</td>
<td>243 ± 6</td>
<td>304 ± 21</td>
<td>362 ± 17</td>
</tr>
<tr>
<td>B</td>
<td>242 ± 6</td>
<td>274 ± 18</td>
<td>368 ± 17</td>
</tr>
<tr>
<td>C</td>
<td>276 ± 3</td>
<td>412 ± 15</td>
<td>490 ± 23</td>
</tr>
<tr>
<td>D</td>
<td>291 ± 5</td>
<td>305 ± 27</td>
<td>457 ± 18</td>
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Body weights of rats in the four groups A, B, C and D were determined at three time points during the 13-week experimental protocol, namely at the start of the protocol, just before surgery and just before death. Group A was fed ethanol for 13 weeks; B was given Lieber–DeCarli isocaloric control diet by pair-feeding for 13 weeks; C was given AIN-93M ad libitum diet for 13 weeks; D was given the same ethanol diet as A for 6 weeks and, after surgery, was switched to ad libitum feeding of the Lieber–DeCarli control diet. The average gains in body weight per week for all four groups between the three time points are also given in this Table. Data are reported as mean ± 95% confidence interval for each group.

*Greater than A and B ($P < 0.001$); †greater than A and B ($P < 0.005$); ‡no difference among A, B and D, but these were less than C ($P < 0.001$); ‡greater than A and B ($P < 0.003$) and less than C ($P < 0.001$).
of hepatomegaly, the livers from ethanol-fed rats in group A showed a 6–9-fold elevation in CYP 2E1 activity compared with the other three groups (Table 2). Steatosis was also evident in these animals, as indicated by 2.0–2.4-fold higher levels in hepatic triglycerides \( (P \leq 0.007) \). The cessation of ethanol consumption by the animals in group D after surgery resulted

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver weight (g liver/100 g body weight)</th>
<th>Hepatic protein content (mg protein/100 g body weight)</th>
<th>CYP 2E1 activity (nmol/min/mg protein)</th>
<th>Triglyceride (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3.25 ± 0.13</td>
<td>583.94 ± 285.68</td>
<td>0.702 ± 0.18*</td>
<td>50.73 ± 26.61*</td>
</tr>
<tr>
<td>B</td>
<td>3.18 ± 0.17</td>
<td>568.69 ± 365.79</td>
<td>0.114 ± 0.03</td>
<td>24.37 ± 30.36</td>
</tr>
<tr>
<td>C</td>
<td>3.24 ± 0.17</td>
<td>490.00 ± 104.21</td>
<td>0.088 ± 0.12</td>
<td>20.90 ± 13.38</td>
</tr>
<tr>
<td>D</td>
<td>3.21 ± 0.1</td>
<td>501.40 ± 35.25</td>
<td>0.073 ± 0.07</td>
<td>20.23 ± 13.79</td>
</tr>
</tbody>
</table>

Liver weight, liver protein content, cytochrome \( P450 \) 2E1 (CYP 2E1) activity and hepatic triglyceride content were determined in groups A, B, C and D at the conclusion of the 13 week protocol. Group notation is the same as in Table 1. Mean (± 95% confidence interval) values for the four groups are presented.

*The results for CYP 2E1 and triglyceride content in ethanol-fed rats in group A were significantly different from each of the other three groups. Comparisons among groups B, C and D revealed no significant differences. There were no significant differences in weight or protein content of the liver among the four groups.
in CYP 2E1 activity and triglyceride levels that were equal to those of groups B and C.

Outcome of bone repair

Bending rigidity of fibulae from ethanol-fed rats (group A) and the pair-fed controls (group B) had similar values, which were 48% (P < 0.001) and 47% (P < 0.001) lower than that of rats fed the AIN-93M diet ad libitum (group C) (Fig. 1). Fibulae in group D rats, which were fed the ethanol diet during the 6 weeks before surgery and the Lieber–DeCarli control diet ad libitum thereafter, had mean rigidity comparable to group C (Fig. 1). The flexural modulus (a material property) of repair tissue in ethanol-fed rats (group A) was 55% lower (P = 0.046) than in their pair-fed controls (group B) (Fig. 2). The mean area of cross-section of the fibulae at the bend test site in ethanol-fed rats (group A) was 30% larger (P = 0.006) than that in pair-fed controls (group B) and 28% larger (P = 0.008) than that in group D (Table 3). The area of cross-section in group B was 14% less (n.s.) than in the control rats that were fed the standard maintenance diet (group C). There were analogous differences in area moment of inertia among the four groups (Table 3), but these differences were not statistically significant. The mineral contents of the repair tissues in ethanol-fed rats (group A) and their pair-fed controls (group B) were 16% (P < 0.001) and 13% (P = 0.001) less than group C (Fig. 3). There was no deficiency in the mineral content of repair tissues in group D, compared to group C.

DISCUSSION

The main finding in this study was that the bone repair tissue in the ethanol-fed rats in group A was inferior to that in the pair-fed controls (group B) in material properties, as indicated by a significantly lower flexural modulus. Thus, 13 weeks of continuous ethanol consumption had an adverse effect on the bone repair process in this experimental model. As discussed below, it is unlikely that this result can be explained by differences in either the amount of food consumed or in the gain of body weight during the protocol.

The most obvious effect of the reduced food consumption by rats in groups A and B is the lower rates of gain of body weight by these animals, compared with those in groups C and D (Table 1, last column). We recognized that the pattern of differences in rigidity of fibula among the four groups (Fig. 1) closely resembled that of the average body weights of these animals at death (Table 1, column 4). Therefore, we examined whether the results for rigidity can be explained on the basis of differences in gain of body weight or the amount of food consumed. In a previous study in our laboratory using this bone repair model in chow-fed male Sprague–Dawley rats, which weighed 330–360 g at death, the rigidity was comparable (1.60 ± 0.42 · 10^-3 Nm^2, unpublished data) with that of group C (Fig. 1). Since final body weights of rats in groups A and B in the present study were similar to the previous study, the lower rigidity of fibula in these rats compared with those of group C cannot be attributed to their reduced gain of body weight during the 13-week protocol.

We also examined whether the lower flexural modulus in the ethanol-fed rats in group A compared with pair-fed controls (group B) can be explained in terms of factors other than ethanol. First, there was no difference between these two groups in their average body weights at the start or at the end of the 13-week protocol. Thus, group B proved to be a valid...
control for the lower body weight gain of ethanol-fed rats in group A associated with their reduced food consumption. In group B, despite the reduced consumption of the Lieber–DeCarli control diet imposed by pair feeding and the resultant retardation in body weight gain, the flexural modulus of repair tissue was comparable to that of group C. Although group C received the AIN-93M diet, rather than the Lieber–DeCarli control diet, the latter is considered to be comparable to the former in nutrition. Indeed, the flexural modulus in group D, which received the Lieber–DeCarli control diet ad libitum after surgery, is nearly the same as in group C. Therefore neither the reduced food consumption nor the lower body weight at death is a reasonable explanation of the deficiency in flexural modulus of the repair tissue in rats in group A. Thus, we conclude that ethanol was the most likely cause of this deficiency.

The geometric properties of the repaired fibula were also influenced by ethanol consumption. The area of cross-section at the midpoint of the repair site in the fibula of rats in group A was significantly larger than that in group B (Table 3). The area moment of inertia was also larger in group A, but the difference was not statistically significant. Nevertheless, numerically, these results provide a partial explanation for the rigidity of fibula in group B to be the same as group A, in spite of the larger flexural modulus, because of the relationship in equation 4 above. These results suggest a trend in the bone repair process to compensate partially for the inferior mechanical properties of the repair tissue by increasing its bulk as measured by area of cross-section. This, then, would have the effect of mitigating the deficiency in mechanical properties of the bone as a whole and hence in its function.

Comparison of the present study with earlier studies of fracture healing in ethanol-fed rats (Janicke-Lorenz and Lorenz, 1984; Pierce and Perry, 1991; Nyquist et al., 1999) may not be valid because of the difference in the method of ethanol feeding. In the earlier studies, rats were fed laboratory chow ad libitum and given 15–20% ethanol in the drinking water over periods of 1 year, 8 days and 5 weeks, respectively, before creating the bone injury. If we ignore this difference, the study of Nyquist et al. (1999) comes closest to ours in the duration of feeding before and after bone injury. Although they found significant differences in bending rigidity and strength between ethanol-fed and control rats, they concluded that ethanol had no effect in fracture healing, since they also found similar differences for the non-fractured tibia. However, it was noted that the presence of a bone injury in the rat is known to influence new bone formation at distant skeletal sites (Mueller et al., 1991). The study design of Nyquist et al. (1999) does not allow a determination of these effects. Therefore, in view of the statistically significant differences in bending rigidity and strength which they found for fractured tibia, between ethanol-fed and control groups, an effect of ethanol specifically on the fracture healing process in their study cannot be ruled out.

The mineral content of the repair tissue in the pair-fed control rats (group B) was significantly less than in rats receiving the AIN-93M diet ad libitum for 7 weeks after surgery (group D) (Fig. 3). Although there was a further decrease due to ethanol (i.e. from group B to A), it was not statistically significant. In chow-fed rats given ethanol in drinking water, Pierce and Perry (1991) found the mineral content of fractured bones to be nearly the same as in controls not receiving ethanol. These findings suggest that ethanol consumption does not diminish the mineral content of the newly formed tissue in the repair site. However, if ethanol interferes with osteoblast function (Dyer et al., 1998), it is likely to disrupt the synthesis of an ossifiable matrix resulting in a maldistribution of the mineral. In other words, if dystrophic mineralization (Nimni et al., 1988), rather than ossification of the newly synthesized matrix, occurs, material properties (e.g. flexural modulus) will be diminished without a decrease in mineral content.

Although the relationship between liver disease and bone disease in alcoholism is not well established, more severe liver disease appears to be associated with greater bone loss (Lalor et al., 1986; Diamond et al., 1989). In the present study, we observed mild changes in liver, due to alcohol consumption (Table 2). The 4- to 5-fold elevation in CYP 2E1 activity in livers of ethanol-fed rats confirmed results reported by others, demonstrating that chronic ethanol consumption induces the P450 2E1 isozyme (Buhler et al., 1992; Akihiko et al., 1995; Hu et al., 1995; Wu and Cederbaum, 1996). The 2.0–2.4-fold higher levels of hepatic triglycerides in these animals also confirmed previous observations (Donehue et al., 1987; Guzman and Castro, 1990). Cessation of ethanol consumption during the 7-week post-surgery period (group D) resulted in normalization of the liver along with the normalization of the bone repair outcome, suggesting a relationship between pathological states in liver and in the bone repair process. Therefore, further studies are warranted using an experimental model in which liver damage is more evident. The intragastric feeding model of Tsukamoto et al. (1985a,b), or the low-carbohydrate oral diet devised by Lindros and Jarvelainen (1998), could be well suited for this purpose, since these models produce more serious liver damage. Either of these models could be used to investigate whether there is a link between liver damage and faulty bone repair.

In conclusion, we found that 13 weeks of feeding ethanol as part of a nutritionally adequate liquid diet (during 6 weeks before and 7 weeks after bone injury) resulted in significant deficiency in the outcome of bone repair compared to ad libitum feeding of a standard maintenance diet. The specific effect of ethanol, apart from any effect associated with reduced food consumption, was to produce repair tissue with inferior material properties (flexural modulus). Cessation of ethanol consumption after bone injury along with ad libitum feeding of the liquid control diet brought about a normal outcome of bone repair.

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