Table 1. ADH activity in livers of adult male and female animals of different species*

| Species | ADH activity (μmoles/g liver/hr) | | (mmoles/kg body wt/hr) | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| Mouse | | | | |
| C57BL/6 | 459.0 ± 7.2 | 547.2 ± 15.0† | 24.89 ± 0.52 | 28.24 ± 0.78‡ |
| DBA/2 | 316.2 ± 10.2 | 371.4 ± 4.8‡ | 16.35 ± 0.24 | 20.22 ± 0.52† |
| Rat | | | | |
| Sprague–Dawley | 292.2 ± 10.8 | 312.0 ± 18.6 | 8.46 ± 0.46 | 10.11 ± 0.85 |
| SH strain | 122.6 ± 3.4 | 300.1 ± 16.7§ | 4.54 ± 0.19 | 13.31 ± 0.64‖ |
| Guinea pig | 202.8 ±13.8 | 238.2 ± 13.2 | 8.59 ± 0.54 | 6.46 ± 0.61 |
| Rabbit | 577.8 ± 24.0 | 624.0 ± 9.0 | 16.57 ± 1.37 | 19.11 ± 0.33 |
| Dog | 117.0 ± 14.4 | 137.6 ± 20.4 | 1.59 ± 0.17 | 1.89 ± 0.20 |

* Values represent means ± S.E.M. (N = 4–5 males or females). Only statistically significant differences ($P < 0.05$) in ADH activity between males and females of each species and strain are indicated.
‡ $P < 0.01$.
† $P < 10^{-3}$.
§ $P < 10^{-4}$.
‖ $P < 10^{-5}$.

of NADH in ethanol metabolism across the zoological scale.

In conclusion, it appears that the high activity of liver ADH in adult females relative to adult males in some rodent strains does not constitute a general phenomenon, but it depends on the species and strains of animals studied. In addition, for the species studied, liver ADH levels correlated with reported rates of *in vivo* ethanol metabolism.

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## Effects of dimethyl sulfoxide (DMSO) on bleomycin-induced pulmonary fibrosis

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Bleomycin, an anti-neoplastic agent composed of a heterogeneous mixture of at least thirteen components [1], has been demonstrated to have effective anti-tumor activity in the treatment of squamous cell carcinomas as well as some lymphomas. Since it is less immunosuppressive than most other anti-neoplastic agents, it is often used in a combination drug regimen [2]. Unfortunately, the high incidence of pulmonary toxicity in those patients treated bleomycin impedes cancer therapy and is regarded as the most significant limiting factor for an intensive and prolonged course in bleomycin cancer therapy [3].

The pulmonary toxicity is characterized by the excessive deposition of connective tissue. Collagen, a major component of connective tissue, has been the focus of many

studies of bleomycin-induced pulmonary fibrosis [4–6]. A single intratracheal (i.t.) injection of 1.5 units of bleomycin will produce fibrosis, which can be estimated by quantitative measurements of lung collagen content.

To prevent the development of pulmonary fibrosis in response to bleomycin therapy, many patients are treated chronically with glucocorticoids [7]. Unfortunately, a high percentage of patients are unresponsive to the glucocorticoid treatment and/or experience serious steroid-related side-effects [8].

In these studies, the ability of dimethyl sulfoxide (DMSO) to inhibit bleomycin-induced pulmonary fibrosis was investigated. DMSO has been reported to have anti-inflammatory activity as well as an inhibitory effect on collagen deposition [9, 10]. The mechanism by which DMSO produces these effects is unknown. Fox and Fox [11] suggested that DMSO has anti-inflammatory activity because it has the ability to act as a free-radical scavenger. Gries *et al.* [12] and Middleton *et al.* [13], however, suggested that DMSO inhibited excess collagen deposition by solubilizing collagen and removing it from the area. Since intratracheal administration of bleomycin causes both lung inflammation and collagen deposition, the present experiments were conducted to determine the ability of DMSO to prevent these changes.

*Materials and methods*

Male Sprague–Dawley rats (Cesarean-derived, barrier-restrained) weighing 175–200 g were obtained from Charles River Laboratories, Wilmington, MA. They arrived in filtered boxes and were kept isolated from other laboratory animals. The animals were housed one treatment group per filter-top cage and received tap water and laboratory chow *ad lib*.

Following anesthesia with pentobarbital (35 mg/kg), one-half of the animals were given a single i.t. injection of 0.3 ml saline. The rest of the animals received 1.5 units of bleomycin sulfate (Bristol Laboratories, Syracuse, NY) [14, 15], dissolved into 0.3 saline.

The rats given bleomycin were divided into two groups. Beginning on day 1, one group was given eight daily injections of saline i.p. while the remaining group was given daily i.p. injections of DMSO, 5 g/kg. A 50% dilution of chromatographically pure DMSO (Sigma Chemical Co., St. Louis, MO) was used for all injections. The rats given saline intratracheally were also divided into two groups with one group being given eight daily injections of 2.0 ml saline while the remaining group was given eight daily i.p. injections of DMSO (5 g/kg).

Twenty-four hours after the last i.p. injection, the animals were killed by decapitation and lungs were rapidly removed, rinsed in a cold (2°) Krebs–Ringer bicarbonate solution (pH 7.4) to remove blood, and minced with scissors. The lung minces were homogenized in 0.9% NaCl using a co-axial glass homogenizer packed in ice. Separate aliquots of the whole homogenate were taken for analysis of DNA [16], protein [17] and collagen [18] content.

All data were analyzed for statistical significance using the Duncan's multiple range test and the Student–Newman–Keuls test [19].

*Results*

At the end of the 8-day experimental period, the animals were killed and their lungs were rapidly removed. The lungs of each animal were grossly inspected for the presence of hemorrhagic lesions. The lungs of the control animals, which received no drugs, were pink and clear of any lesion except for one animal which had a few small pinpoint lesions in one lung. The lungs of DMSO-treated animals were visually no different from controls. All of the animals treated with bleomycin had multi-focal hemorrhagic lesions involving a considerable amount of lung tissue; however, administration of DMSO to rats treated with bleomycin resulted in a reduction of the severity of hemorrhagic lesions observed when animals received bleomycin alone.

Rats given 1.5 units of bleomycin weighed significantly less (50%) than controls 1 week after instillation (Table 1). When DMSO was given daily for 8 days to bleomycin-treated animals, body weights were only 20% less than controls. Rats given DMSO alone weighed slightly less (10%) than control animals but this difference was not significant. The lungs from the bleomycin-treated rats were elevated significantly in both total wet weight and DNA content. Administration of DMSO to bleomycin-treated rats appeared to prevent the previously seen changes in lung wet weight and DNA content. When DMSO was given by itself, there appeared to be no effect on either variable.

The collagen and protein contents of lungs from bleomycin-treated rats, reported as per lung or per mg DNA, were elevated significantly over control values (Table 2). This has been reported by several investigators [4–6, 20]. When bleomycin animals were given DMSO, the protein and collagen contents of the lungs were not different from control. DMSO given by itself did not alter either variable from control levels.

*Discussion*

The toxicity of bleomycin-induced pulmonary fibrosis is well documented. As seen in our results, intratracheal administration of bleomycin caused hemorrhagic lesions in the lungs and increased total lung weight. Biochemically, the lungs were characterized as having increased DNA, protein and collagen. These results are similar to the type of changes observed in man following bleomycin usage [21] and have been documented in several animal studies [5, 14, 20].

The purpose of the present studies was to investigate the ability of DMSO to prevent bleomycin-induced lung fibrosis. DMSO has been reported to have anti-inflammatory activity, and to alter collagen deposition in different disease states. In the present experiments, a DMSO dose of 5 g/kg was used as a maximum non-toxic dose. Cumulative effects or increased toxicity have not been reported to occur in rats with repeated daily i.p. administration when the daily dose did not exceed the single maximum tolerated dose of 5 g/kg and the concentration of DMSO injected remained less than or equal to 50% [22].

A significant decrease in body weight was the first observable sign of bleomycin toxicity. Animals receiving bleomycin had body weights that were 40–50% less than controls. Although DMSO-treated animals weighed approximately 10% less than controls, the body weights of animals treated with both compounds were significantly higher than those of animals treated with bleomycin alone (Table 1).

Since total lung DNA was elevated, there appeared to be increased cellularity in the damaged lungs. DMSO, when given by itself, caused some increase in lung weight, but did not alter any of the measured biochemical variables. As a further indication of cellular damage, the lungs from bleomycin-treated rats had increased amounts of both total protein and collagen.

The biochemical mechanisms for bleomycin-induced pulmonary fibrosis are not clearly understood. However, bleomycin has been reported to generate superoxide radicals [23] as well as highly reactive hydroxyl radicals [24] which presumably cause DNA strand breakage [25, 26] and lipid peroxidation [27]. Trush *et al.* [28], in an attempt to test this hypothesis, has reported that reactive oxygen scavengers, superoxide dismutase and dimethylurea, inhibit bleomycin-mediated DNA chain breakage.

When bleomycin animals were treated with DMSO, there was complete protection. All biochemical variables measured were not different from control. However, the mechanism by which DMSO is protecting against the bleo-

Table 1. Effect of DMSO on body weight, gram lung weight per gram body weight and DNA content in lungs of bleomycin-treated rats

| Group* | Body wt (g) | $\frac{\text{Gram lung weight}}{\text{Gram body weight}} \times 100$ | DNA ($\mu$g/lung) |
|---|---|---|---|
| Control | 255 ± 12† (4) | 0.44 ± 0.01 (4) | 7389 ± 241 (4) |
| Bleomycin | 135 ± 8‡ (4) | 1.38 ± 0.10‡ (4) | 9137 ± 627‡ (4) |
| DMSO | 226 ± 3 (7) | 0.45 ± 0.01 (7) | 6722 ± 257 (7) |
| Bleomycin/DMSO | 201 ± 18‡ (4) | 0.62 ± 0.10 (4) | 7537 ± 436 (4) |

* Control: a single intratracheal instillation of 0.3 ml saline followed by 2 ml saline i.p. daily, for 8 days.
Bleomycin: a single i.t. instillation of 0.3 ml bleomycin (1.5 units/rat), followed by 2 ml saline i.p. daily, for 8 days.
DMSO: a single i.t. instillation of 0.3 ml saline, followed by DMSO (5 g/kg) i.p. daily, for 8 days.
Bleomycin/DMSO: a single i.t. instillation of 0.3 ml bleomycin (1.5 units/rat), followed by DMSO (5 g/kg) i.p. daily, for 8 days.
† $\bar{X}$ ± S.E. (N).
‡ $P < 0.05$ when compared to control animals.

Table 2. Effect of DMSO on protein and hydroxyproline content in lungs of bleomycin-treated rats

| Group* | Protein (mg/lung) | Protein (mg/mg DNA) | Hydroxyproline ($\mu$g/lung) | Hydroxyproline ($\mu$g/mg DNA) |
|---|---|---|---|---|
| Control | 156 ± 8† (4) | 21.16 ± 0.74 (4) | 1999 ± 228 (4) | 273 ± 34 (4) |
| Bleomycin | 289 ± 23‡ (4) | 31.63 ± 1.24‡ (4) | 3528 ± 179‡ (4) | 388 ± 23‡ (4) |
| DMSO | 136 ± 5 (7) | 20.24 ± 0.65 (7) | 1516 ± 88 (7) | 226 ± 13 (7) |
| Bleomycin/DMSO | 155 ± 17 (4) | 20.57 ± 0.98 (4) | 1734 ± 156 (4) | 229 ± 12 (4) |

* See Table 1.
† $\bar{X}$ ± S.E. (N).
‡ $P < 0.05$ when compared to control animals.

mycin-induced lung fibrosis is not known. DMSO has been reported to have a wide range of pharmacological activities. Several studies have suggested that DMSO is an anti-inflammatory agent [9, 10] which has the ability to suppress edema formation [29]. Berliner and Ruhmann [30] have demonstrated that DMSO will inhibit the growth of fibroblasts *in vitro*, while others [12, 13] have suggested that there is an increased solubilization of collagenous fibers in the presence of DMSO. DMSO does not appear to affect elastic fibers in tissues [31]. Recently, Fox and Fox [11] have suggested that the anti-inflammatory effects of DMSO may be related to its ability to act as a free radical scavenger.

Whichever mechanism is proven to be correct, the data presented in these studies clearly demonstrate that DMSO can protect against acute bleomycin-induced lung toxicity. Currently, clinical treatment regimens of corticosteroids have been used in most patients in an attempt to reduce the inflammatory response to bleomycin therapy [32]. However, many patients were either unresponsive to steroid therapy [7] and/or exhibited a wide variety of deleterious effects [8]. Therefore, the development of a more effective treatment is needed. Since DMSO has the ability to protect against acute bleomycin-induced lung fibrosis and has few reported side-effects [33], additional studies concerning the ability of various doses of DMSO to prolong animal survival following bleomycin would be of interest. These data would also help to establish the mechanism of action of DMSO in preventing lung fibrosis.

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# Inhibitory effect of chlorpromazine on bone formation *in vivo* and *in vitro*

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Chlorpromazine (CPZ), a phenothiazine derivative, is a potent tranquillizing agent for treatment of psychiatric disorders. However, CPZ is known to occasionally cause toxic reactions such as hepatitis with jaundice, hypoplastic anaemia and dermatitis [1–3]. Moreover, growth retardation and malformation in the offspring of CPZ-treated pregnant animals have also been demonstrated [4–6]. However, the precise mechanism of CPZ action on skeletogenesis is not known.

The present study was therefore undertaken to investigate the effects of CPZ on bone formation using rats and clonal osteoblastic MC3T3-El cells, which have retained a wide variety of osteoblastic cell functions [7, 8]. We now report that CPZ lowers alkaline phosphatase (EC 3.1.3.1) activity in rat calvaria more than that in the liver and duodenum *in vivo* CPZ also specifically decreases alkaline phosphatase activity and collagen synthesis in these cells.

## *Materials and methods*

Eight-week-old male rats of the Wistar strain weighing about 200 g body wt were used throughout. Various doses of CPZ dissolved in 0.3% or less than 0.3% methyl alcohol (final concentration) was injected intraperitoneally. After appropriate periods of treatment, rats were killed and calvaria, liver and duodenum were quickly removed. These organs were weighed and homogenized in 5 vol. of 10 mM Tris–HCl (pH 8.4) containing 0.2 M lithium 3,5-diiodosalicylate, 0.2% Triton X-100, and 20% butanol at 4° for 2 min in a Waring blender. The homogenate was centrifuged at 20,000 *g* for 20 min.

For *in vitro* experiments, $5 \times 10^4$ clone MC3T3-El cells were plated in 35-mm Falcon plastic dishes in 2 ml of $\alpha$-minimum essential medium ($\alpha$-MEM, Flow Laboratories, Rockville) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana), cultured for 3 days, and then transferred to medium containing 10% serum plus various concentrations of chlorpromazine hydrochloride (CPZ, Sigma Chemical Co., St. Louis). After appropriate periods of cultivation, cells were washed three times, scraped into 2 ml of 0.2% Nonidet P-40 containing 1 mM $MgCl_2$, and sonicated for 5 min with a sonifier cell disruptor (Model UCD-100, Tosho, Yokohama, Japan). The sonicates were centrifuged for 10 min at 3000 rpm, and the supernatants were used for the enzyme assay. Alkaline phosphatase activity in the organs or the cells was assayed by the method of Lowry *et al.* [9], with *p*-nitrophenyl phosphate as substrate. DNA content was measured by a fluorometric method [10], and protein content was estimated by the method of Bradford [11].

Cells in culture were treated with various concentrations of CPZ for 24 hr, and then media were replaced with 1 ml of $\alpha$-MEM containing 50 $\mu$g each of ascorbic acid and $\beta$-aminopropionitrile, and labelling with 10 $\mu$Ci of [$^3$H]-proline (L-3[3,4$^3$H], 20–30 Ci/mmole, New England Nuclear Co., Boston, MA) was conducted for 3 hr. The cell suspension was treated with 10% trichloroacetic acid and 0.5% tannic acid (final concentrations). After centrifugation, the precipitates were washed three times with the same solution and twice ice-cold acetone. Collagenase-digestible protein and non-collagen protein were determined according to the procedures of Peterkofsky and Diegelmann [12].

## *Results and discussion*

First the effects of CPZ on bone tissues *in vivo* was examined by a single injection of 10 mg/kg CPZ into rats. Alkaline phosphatase activity in calvaria of CPZ-treated rat began to decrease at 6 hr and reached a minimum at 17 hr after its injection. CPZ affected calvaria to a greater extent and for a longer period than liver and duodenum. Although the activity in liver began to recover by 40 hr, that in calvaria did not. The effects of CPZ were more specific for calvaria than for liver and duodenum, with the most decreased activities being 18, 51 and 77%, respectively, of controls at 17 hr after treatment (Fig. 1). These results suggest a rather specific inhibitory effect of CPZ on bone tissues *in vivo*

Next, the possibility of effects of CPZ was examined on osteoblastic clone MC3T3-El cells *in vitro*. DNA and protein contents increased slightly at lower concentrations of CPZ, but protein content decreased at 10 and 20 $\mu$g/ml CPZ, in clone MC3T3-El cells (Table 1). CPZ caused a decrease in alkaline phosphatase activity in a dose-related fashion; the enzyme activity was significantly decreased by 5 $\mu$g/ml CPZ and became one-half that of the control at 10 $\mu$g/ml. The enzyme activity was decreased further at 20 $\mu$g/ml, at which concentration the effect was greater for the activity than for DNA and protein contents. These results indicate that the effect of CPZ is more specific for osteoblastic cell function than for general cytotoxicity.