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Abstract

Effects of antioxidants, such as superoxide dismutase, vitamin C, vitamin E, 4-(0-benzylphenoxy)-N-methylbutylamine hydrochloride (bifemelane), and selenite on survival of adult rat hepatocytes were examined under normoxic and hyperoxic conditions in serum-free primary culture. The tested antioxidants, except for vitamin C, significantly increased the survival rate of hepatocytes under the normoxic condition (under air). Thus, even the normoxic culture condition is hyperoxic for hepatocytes. Elevation of oxygen tension (40% O2) caused severe morphologic degeneration of hepatocytes and remarkable decrease in the survival rate of the cells. Addition of the antioxidants effectively protected hepatocytes from the morphologic degeneration, and significantly improved the survival of the cells under the hyperoxic condition. These findings indicate that the antioxidants can maintain the long-term survival of hepatocytes in serum-free primary culture.

KEYWORDS: oxugen tension, hepatocytes, serum-free primary culture, survival, antioxidants

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— Brief Note —

Effects of Antioxidants on Survival of Adult Rat Hepatocytes under Various Oxygen Tensions in Serum-Free Primary Culture

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Effects of antioxidants, such as superoxide dismutase, vitamin C, vitamin E, 4-(0-benzylphenoxy)-N-methylbutylamine hydrochloride (bifemelane), and selenite on survival of adult rat hepatocytes were examined under normoxic and hyperoxic conditions in serum-free primary culture. The tested antioxidants, except for vitamin C, significantly increased the survival rate of hepatocytes under the normoxic condition (under air). Thus, even the normoxic culture condition is hyperoxic for hepatocytes. Elevation of oxygen tension (40 % O_2) caused severe morphologic degeneration of hepatocytes and remarkable decrease in the survival rate of the cells. Addition of the antioxidants effectively protected hepatocytes from the morphologic degeneration, and significantly improved the survival of the cells under the hyperoxic condition. These findings indicate that the antioxidants can maintain the long-term survival of hepatocytes in serum-free primary culture.

Key words : **oxygen tension, hepatocytes, serum-free primary culture, survival, antioxidants**

Reactive oxygen species can react with almost any cellular components. Their interaction with lipid components in cellular membranes disrupts the structure of the membranes by peroxidation, resulting in a loss of integrity (1) and even cell death (2).

Even under normoxic conditions, *i. e.*, under air, cultured cells are exposed to reactive oxygen species generated by mitochondrial respiration (3); light exposure of media containing tyrosine, tryptophan and riboflavin (4); and autoxidation of cysteine in some media (5). Thus we examined effects of antioxidant compounds [vitamin C (6), vitamin E (7), 4-(0-benzylphenoxy)-N-methylbutylamine hydrochloride (bifemelane) (8), and selenite (9)] and an antioxygenic enzyme, superoxide dismutase (SOD), on survival of adult rat hepatocytes under normoxic and hyperoxic conditions in serum-free primary culture.

Donryu male rats (3 months old) inbred in this laboratory were used in the present experiments. As reported previously, hepatocytes were isolated from normal adult rats by the liver perfusion technique with collagenase type I (Sigma Chemical Co., St. Louis, MO, USA) (10). Their initial viability was 85 to 95 % as measured by trypan blue exclusion. DM-160 medium (Kyokuto Pharmaceutical Industrial Co., Ltd.,

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Tokyo, Japan) supplemented with penicillin at 100 U/ml, streptomycin at 100 μg/ml, and fungizone at 1 μg/ml was used as basal medium. The hepatocytes suspended in the basal medium were inoculated at a cell number of 1.4 x 10^6 cells in 4 ml of medium onto 60-mm Falcon plastic dishes coated with rat tail collagen prepared as previously described (11). The cells were cultured in a 37 °C humidified incubator, under the following gas phases: 1) 95 % air and 5 % CO_2, 2) 10 % O_2, 85 % N_2, and 5 % CO_2, and 3) 40 % O_2, 55 % N_2, and 5 % CO_2. The mixed gases were made using a gas proportioner (Ikemoto Rika Kogyo Co., Ltd., Tokyo, Japan). To enhance attachment efficiency of the inoculated hepatocytes, dexamethasone sodium phosphate (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan) and insulin (Sigma) were added to the cultures only during the first 24 h at concentrations of 10 μM and 10 μg/ml, respectively (12).

Vitamin C, seleneous acid, bifemelane (a gift from Eisai Co., Ltd., Tokyo, Japan), and SOD (Sigma) were directly dissolved in the basal medium at the final concentrations indicated in the text. Vitamin E was dissolved in ethanol, and then added to the basal medium to give the indicated concentrations, with 0.1 % ethanol concentration. After the 24-h attachment period, treatment of the hepatocyte primary cultures with the antioxidants was started and continued for 6 days. The culture media containing various antioxidants were renewed every 2 days. Some cultures were treated with or without 0.1 % ethanol for control in the same way.

For determination of surviving hepatocyte number, the hepatocytes cultured on the rat tail collagen-coated dishes were dispersed with 0.02 % EDTA, 0.1 % trypsin (1: 250, Difco, Detroit, MI, USA) and 0.05 % collagenase, as previously described (11). The number of viable hepatocytes was determined by trypan blue exclusion in a hemacytometer and expressed as the mean of three dishes. For statistical evaluation, the data were analyzed using Student's *t*-test. The level of significance was chosen at $P < 0.05$.

The antioxidants, except for vitamin C, significantly increased the survival rate of hepatocytes under the normoxic condition (20 % O_2) in primary culture (Fig. 1). Suleiman and Stevens (13) have shown that the ordinary normoxic culture condition (under air) is hyperoxic for hepatocytes, and that the generation of reactive oxygen species is elevated as the oxygen tension increases. Actually, our present experiments revealed that the high oxygen tension (40 % O_2) caused severe morphologic degeneration of hepatocytes including enlargement of cell surface and multinucleation (Fig. 2 A), resulting in a marked decrease in the survival rate of the cells (Fig. 1). Addition of the antioxidants protected

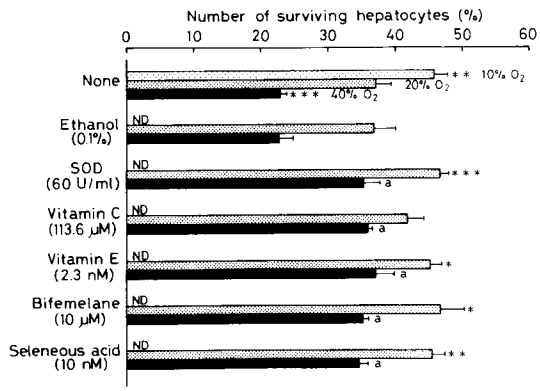

Fig. 1 Effects of oxygen tension and antioxidants on survival of hepatocytes in serum-free primary culture. Hepatocytes inoculated at 1.4 x 10^6 cells/60-mm dish were cultured either under 10 % O_2 (a mixture of 10 % O_2, 85 % N_2, and 5 % CO_2), 20 % O_2 (a mixture of 95 % air and 5 % CO_2), or 40 % O_2 (a mixture of 40 % O_2, 55 % N_2, and 5 % CO_2). Treatment of the cultures with or without various antioxidants at each optimal concentration was started after 24-h attachment period and continued for 6 days. Some cultures were treated with 0.1 % ethanol in the same way as a control for vitamin E treatment. The viability of hepatocytes cultured for 1 week was expressed as percentages of the number of the cells attached 24 h after the initiation of cultures, which was considered as 100 %. Results are expressed as the mean ± SD of three dishes. Asterisks and a letter denote significant differences against control (none or 0.1 % ethanol) under 20 % O_2 and 40 % O_2, respectively, as follows: *, $P < 0.025$; **, $P < 0.01$; *** and *a*, $P < 0.005$. ND, not determined.

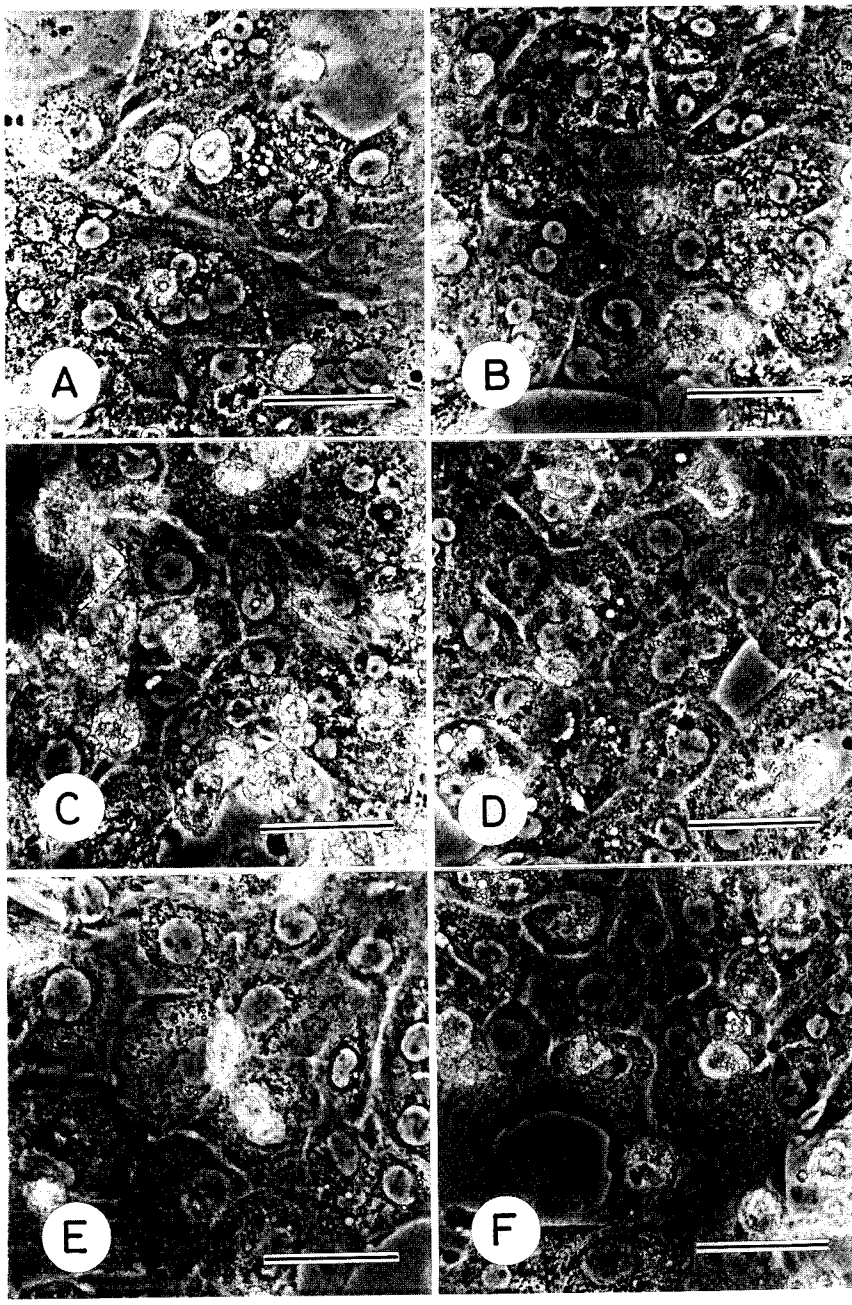

Fig. 2 Appearances of hepatocyte primary cultures on week 1 in the presence or absence of antioxidants under the hyperoxic condition (40 % O_2) in serum-free primary culture. The hepatocyte primary cultures were treated with (*B-F*) or without (*A*) SOD (60 U/ml, *B*), vitamin C (113.6 μM, *C*), vitamin E (2.3 nM, *D*), bifemelane (10 μM, *E*), or seleneous acid (10 nM, *F*) for 6 days after 1-day attachment period. The control culture (*A*) displayed severe morphologic degeneration of hepatocytes, including enlargement of cell surface and multinucleation. All the added antioxidants protected hepatecytes from such morphologic degeneration. Vitamin E was the most effective among the tested antioxidants. Bars indicate 50 μm.

hepatocytes from the morphologic degeneration observed in the control cultures (Fig. 2 B-F), and significantly improved the survival of the cells under the hyperoxic condition (Fig. 1). Conversely, the reduction of oxygen tension (10 % O_2) in the primary culture increased the survival rate of hepatocytes (Fig. 1).

Mitochondria mainly produce reactive oxygen species, such as superoxide and hydrogen peroxide, in a variety of tissues (3). Reactive oxygen species are generated in the two mitochondrial components, the NADH dehydrogenase complex and the ubiquinone-cytochrome *b* region (14). Electron transport inhibitors, such as rotenone and amobarbital, have been reported to inhibit the formation of active oxygen species (15). These inhibitors also effectively increased the survival rate of hepatocytes in serum-free primary culture (date not shown). Thus it is obvious that the reduction of oxidative stress by antioxidants or electron transport inhibitors contributes to longevity of the hepatocytes in serum-free primary cultures.

In conclusion, in serum-free primary cultures, the antioxidants efficiently maintain the long-term survival of hepatocytes under hyperoxic conditions as well as under the normoxic condition.

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