The Effect of L-Ascorbic Acid-2-Phosphate Magnesium on Chicks Injected a Radical Initiator

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Effect of L-ascorbic acid phosphate magnesium (APM) on chicks injected radical initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), was examined. Chicks were fed APM as ascorbate resource for 7 days. Birds were administrated AAPH by intraperitoneal injection after APM feeding. The changes of ascorbate and thiobarbituric acid reactive substances (TBARS) in plasma and liver were determined before, and 3 and 6 hours after AAPH injection. Both plasma and liver ascorbate increased by APM feeding. After AAPH injection, liver ascorbate decreased, but plasma ascorbate increased in both control and APM-fed birds. AAPH administration raised liver TBARS, but decreased plasma TBARS. Both plasma and liver TBARS were lower in APM group than control group before and after AAPH administration. Peroxyl radical trapping activity in plasma was elevated by APM feeding as compared to control group. APM was utilized as ascorbate, and was effective for prevention of oxidative stresses derived from AAPH by increasing plasma and liver ascorbate and plasma radical trapping activity.

Key words : chick, L-ascorbic acid-2-phosphate magnesium, radical initiator, oxidative stress

Introduction

Free radical molecules have been well known to be harmful to animals and to be closely associated with tissue damage and the aging (Ames *et al.,* 1993). In animals, the defense against free radical molecules is provided by protecting reaction of antioxidative enzymes and substances, such as superoxide dismutase, catalase, α-tochopherol, uric acid, glutathione and ascorbic acid (McCord and Fridovich, 1969 ; Burton and Trader, 1990 ; Ames *et al.,* 1988 ; Sies *et al.,* 1992).

L-ascorbic acid-2-phosphate magnesium (APM) is a product by chemical esterification of L-ascorbic acid with phosphate in order to increase the stability of ascorbic acid. The APM has already been put to practical use as a feed additive in fish farming. It has

been reported that APM is hydrolyzed to ascorbic acid in the body of chicken and utilized in the form of ascorbic acid (Aoyagi et al., 1992).

On the other hand, it has been reported that 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) can be used as a peroxyl radical initiator that is automatically pyrolyzed in the body of animals, thus producing radicals which for most part diffuse in organs and tissues, attacking various substrates (Niki, 1992).

The present study was conducted to investigate the effect of AAPH administration on chicks to evaluate adaptability as an animal model for radical injury, and to investigate the effect of a diet admixed with APM on chicks injected AAPH.

Materials and Methods

Experiment 1 : Single-comb White Leghorn male chicks at the age of 7 days were used. Twenty-four birds with medium body weights in the flock were selected, divided into two groups of 12 chicks each, and were individually kept for 7 days in metabolism cages. During the experiment, the chicks in one group (control group) were fed a commercial chick mush (CP 20%, ME 12.1 kJ/g, Showa Sangyo Co. Ltd., Tokyo, Japan) and the other (APM group) were fed the chick mush admixed with 1.5% APM (Showa Denko Co. Ltd., Tokyo, Japan) ad libitum. APM at 1.5% was equivalent to 0.9% ascorbic acid. Light was provided continuously, and ambient temperature was maintained at $30 \pm 2°C$. Body weight and feed intake were measured every day during experiment.

On the last day of the experiment, AAPH (Wako Pure Chemical, Osaka, Japan) was injected intraperitoneally at a dose of 10 mg per 100 g body weight. A preliminary study was performed to assure that under these conditions the mortality of chicks was not lower than 70% for 24 hours after AAPH administration. Just before, and 3 and 6 hours after AAPH injection, three chicks of each group were killed by ether euthanasia and blood and liver were harvested. All the remaining chicks were died within 24 hours of the AAPH injection. Plasma was separated immediately from blood, and plasma and liver were frozen in liquid nitrogen and stored at $-80°C$ until analysis.

Experiment 2 : Six chicks from both the control and APM-treated groups were obtained in the same manner as experiment 1. Blood was collected from the birds, and the ascorbic acid concentration and radical trapping activity (RTA) in the plasma were measured.

Chemical analysis : Ascorbic acid concentration in the plasma and the liver was measured by α,α'-dipyridyl method (Zannoni et al., 1974). Thiobarbituric acid reactive substances (TBARS) of plasma and liver were measured by the methods of Yagi (1976) and of Masugi and Nakamura (1977), respectively. TBARS were expressed as malondialdehyde equivalents. RTA was determined by spectrophotometric method using a methylene blue derivative (10-N-methylcarbamoyl 3,7-bis-dimethylamino 10 H-phenothiazine, MCDP) and AAPH (Sano et al., 1992, Oishi et al., 1985). RTA was expressed as relative values as compared to the mean value of control group.

Statistical Analysis : Values obtained in experiment 1 and 2 were analyzed by general linear model (GLM) procedure (Statistical Analysis Systems, SAS Institute

Inc., Cary, NC) with one-way allocation and Student's t-test.

Results and Discussion

Experiment 1 : Seven-day administration of APM had no effect on body weight gain, feed intake and feed efficiency (body weight gain : feed intake) in chicks.

The changes in plasma ascorbate level in chicks after AAPH injection were shown in Fig. 1. In the control group, 6 hours after AAPH administration, plasma level of ascorbic acid was significantly increased from initial level. In the APM group, ascorbic acid concentration in the plasma was significantly increased from initial level in 3 hours post-drug. In addition, plasma ascorbate level in the APM group was higher than the control group at the pre-drug and 3 hours after AAPH administration.

The change in liver level of ascorbic acid was shown in Fig. 2. In contrast with the results obtained with plasma, liver level of ascorbic acid tended to be decreased after the AAPH injection in the control group. Also in the APM group, ascorbic acid content in the liver was lower in 3 and 6 hours after AAPH administration than pre-drug value. The mean ascorbic acid content in both plasma (366.2 nmol/ml (APM group), 148.2 nmol/ml (control group)) and liver (2.45 μmol/g liver (APM group), 1.77 μmol/g liver (control group)) was significantly higher in APM group compared with control group. These results are in agreement with the previous findings the APM is utilized as ascorbic acid in chicks (Aoyagi *et al.*, 1992). On the other hand, changes in ascorbate
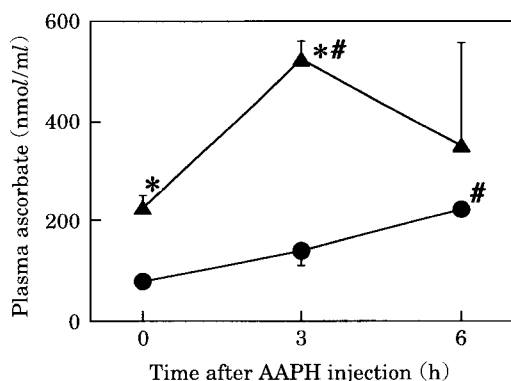

Fig. 1. Time course of the plasma ascorbate concentration in chicks after the administration of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).
●, Control group ; ▲, APM group.
Values are expressed as means±SD (N=3).
*, Significantly different from control group at the same periods (P<0.05).
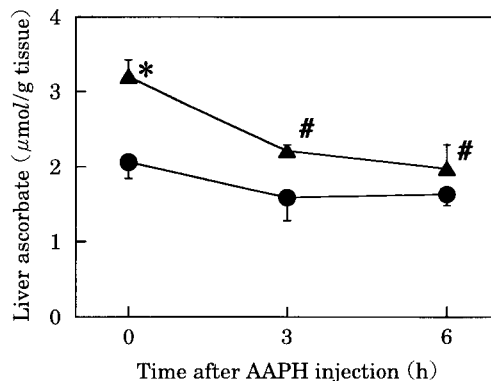#, Significantly different from the initial values in the same group (P<0.05).

Fig. 2. Time course of the liver ascorbate concentration in chicks after the administration of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).
●, Control group ; ▲, APM group.
Values are expressed as means±SD (N=3).
*, Significantly different from control group at the same periods (P<0.05).
#, Significantly different from the initial values in the same group (P<0.05).

in the liver and the plasma were differ from each other. Liver has been considered as a potential contributor to the maintenance of plasma ascorbate (Upston *et al.*, 1999). Increase of plasma ascorbate and decrease of liver ascorbate might be caused by supplementation from the liver to the plasma for protection against oxidative stresses derived from AAPH. However, liver ascorbate was also consumed by protection against radical molecules in the liver itself. To make clear the different change of ascorbate in the liver and the plasma indicated in the present study, the transportation system of ascorbate under oxidative stresses must be investigated. In addition, ascorbate bioproduction was also considered as a reason for increase of plasma ascorbate. We indicated previously the increase of ascorbate in cultured rat hepatocytes under oxidative stress (Sasaki *et al.*, 2001). In rat, ascorbate bioproduction system is expressed in the liver. In chick, however, ascorbate was synthesized in the kidney (Chatterjee, 1973). Investigation about ascorbate synthesis system in the kidney of chicks is also needed for clarification of the increase of plasma ascorbate of chicks administrated with radical molecules.

The concentration of plasma TBARS was expressed in nmol per mg protein, as shown in Fig. 3. A significant decrease in plasma TBARS was observed at 3 and 6 hours after AAPH administration in both control and APM-treated groups. The plasma TBARS at 3 hours after AAPH administration was lower in APM group compared with control group. In addition, the mean values were also significantly lower ($P < 0.05$) in the APM group (24.0 nmol/mg protein) than in the control group (34.3 nmol/mg protein) before, and 3 and 6 hours post-drug.

The liver TBARS are expressed in nmol per mg protein, as shown in Fig. 4. In contrast to the results obtained with the plasma, the liver TBARS tended to be increased with time after AAPH administration in both control and APM groups. The value at 6 hours post-drug was significantly higher in the control group than the pre-drug value. The mean values were significantly lower in APM group (0.341 nmol/mg protein) than in control group (0.399 nmol/mg protein) before, and 3 and 6 hours after AAPH administration. The increase in TBARS in the liver after AAPH injection suggested that oxidative injury was induced by radical molecules derived from AAPH. On the other hand, the decrease in plasma TBARS suggested that the change might be caused by lipid incorporation from the plasma into liver in chicks with hepatic function impairment by AAPH. The change in plasma and liver TBARS were in good agreement of Umegaki *et al.* (1991) that in rats administered carbon tetrachloride, the level of TBARS in liver was incresed, and TBARS in serum was decreased. Furthermore, the lower TBARS in the APM group than in the control group suggested that APM supplementation inhibited lipid peroxidation by AAPH.

Experiment 2 : The APM supplementation to the diet had no effect on body weight gain, feed intake and feed efficiency in chicks, as in experiment 1.

The ascorbate level and relative values of RTA in the plasma of chicks are shown in Table 1. Plasma RTA on peroxyl radicals derived from AAPH in the APM group was remarkably elevated to 6-fold of the control group. Ascorbic acid content in the plasma was high in the APM group, approximately 2.5 times as much as that in the
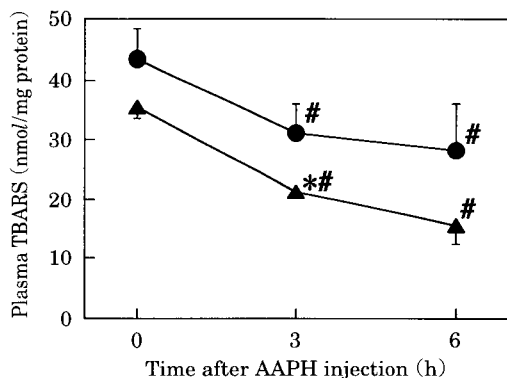
Fig. 3. Time course of the plasma thiobarbituric acid reactive substances (TBARS) concentration in chicks after the administration of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).

●, Control group ; ▲, APM group.
Values are expressed as means±SD (N=3).
*, Significantly different from control group at the same periods (P<0.05).
#, Significantly different from the initial values in the same group (P<0.05).
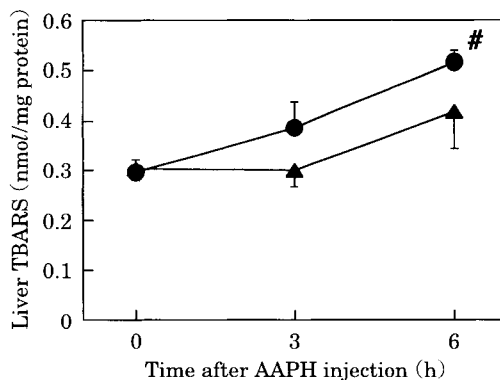
Fig. 4. Time course of the liver thiobarbituric acid reactive substances (TBARS) concentration in chicks after the administration of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH).

●, Control group ; ▲, APM group.
Values are expressed as means±SD (N=3).
#, Significantly different from the initial values in the same group (P<0.05).

Table 1. Effects of L-ascorbic acid-2-phosphate magnesium (APM) feeding on plasma ascorbate concentration and radical trapping activity in chicks

Diet	Ascorbate (n mol/ml)	Radical trapping activity (fold of control value)
Control	62.4[†]	1.0[†]
+APM (1.5%)	197.0*	6.0*
Pooled SEM	14.1	0.06

Values are means of six birds per treatment.
Means with asterisk are significantly different from control value (P<0.01).
[†], One missing value.

control group. It has been pointed out that plasma has generally high antioxidant effect (Stocks and Gutterridge, 1974), whereas in the rat water-soluble RTA is increased after the administration of ascorbic acid (Sano *et al.,* 1992). The present study also showed that radical trapping activity was elevated due to increase in plasma ascorbate level after feeding the APM-containing diet.

Based on the results presented, it may be said that the chick given AAPH as radical initiator can be served as an animal model of radical injury. Furthermore, APM

supplementation is effective for prevention of oxidative stresses induced by AAPH by increasing ascorbic acid concentration in the body of chicks.

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