# RELATIONSHIP BETWEEN ANTIOXIDANT POWER OF PLASMA WITH LIPID PEROXIDE FORMATION IN PLASMA AND LIVER DAMAGES CAUSED BY OVERDOSE OF VITAMIN $K_1$ IN ADULT AND WEANLING RATS

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**Abstract-** In this study the plasma levels of lipid peroxidation (LP) products, protein carbonyls and antioxidant capacity of plasma as judged by ferric reducing ability of plasma (FRAP assay) were compared in adult and weanling rats treated with vitamin $K_1$ (phylloquinone). These factors were differentially affected in the two age-groups following vitamin $K_1$ administration (28, 56, or 84 mg/kg b.w); FRAP activity was unaffected in adults under treatments with vitamin $K_1$, however it was significantly enhanced in immature rats. A remarkable increase in FRAP (~92%) in weanling rats due to high doses of vitamin $K_1$ was associated with a significant suppression (~26%) in plasma LP formation. However, in adults there was a significant increase (~36%) in LP formation due to vitamin $K_1$ treatments. The pattern of changes in plasma protein carbonyls was quite different and unlikely to be related to changes in FRAP. The protective role of induced FRAP was corroborated with histopathological observations carried out on liver tissues obtained from rats treated with 56 mg vitamin $K_1$/kg. Accumulation of fat droplets in hepatocytes was found to be the end point of vitamin $K_1$ treatments to young rats, whereas in adult's livers a number of cell deaths were also observed. The results obtained in the present study suggest that inducible factors in immature rats which contribute to plasma antioxidant activity are probably responsible for prevention of LP formation and protection against oxidative damage caused by high dose of vitamin $K_1$.
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## INTRODUCTION

Vitamin K is a substrate for a liver microsomal enzyme, the vitamin K-dependent carboxylase that catalyzes the posttranslational carboxylation of specific glutamyl residues in a number of proteins to γ-carboxyglutamyl residues (1). This modification is required for the physiological activity of a number of plasma proteins, including the procoagulant factor II, VII, IX and X (2). Vitamin K is a generic term for compounds that include Phylloquinone or Phytonadione (vitamin $K_1$), the Menaquinone series (vitamin $K_2$) and Menadione (vitamin $K_3$). The natural types of vitamin K are vitamin $K_1$ which is synthesized by plants and $K_2$ series which are synthesized by bacteria.

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Vitamin K in the fetus and newborn is maintained at levels less than that necessary to achieve full γ-carboxylation of the K-dependent proteins including those required for homeostasis. Vitamin K has been successfully used in the prevention of hemorrhagic disease of newborn (HDN) (3). In adults, primary vitamin K-deficient states that resulted in bleeding were almost unknown except in the hospital setting where they could readily be diagnosed and monitored by routine coagulation assays. Moreover a controlled state of vitamin K deficiency and associated hypoprothrombinaemia induced by anticoagulant drugs is still seen as beneficial in adults with thromboembolic disease or at risk of it. Therefore in adults, vitamin K deficiency has never been perceived as a threat to health. Vitamin K preparations such as vitamin $K_3$ (menadione) are convenient to use when given orally to pregnant women. However vitamin K levels in the newborn's plasma are much lower than those of the mother, either because of reduced placental transport or due to augmented utilization by the fetus (4). There are different factors that may attribute to the deficiency of vitamin K in newborn

babies. Vitamin K levels are normally 60 times lower in cord blood as compared to that in mother's serum and its level is low in mother's milk. Injection of vitamin $K_1$ to mothers shortly before delivery increases the plasma vitamin K levels significantly but barely crosses the placenta. Accordingly, it is preferable to treat newborns during their first days of birth with a vitamin K preparation. In the neonate, in whom bleeding due to spontaneous vitamin K prophylaxis introduced in the 1950s continued to be common with little public debate or doubts until the 1980s.

Allied to this, intake of vitamin K beyond those required to prevent bleeding have not until recently been perceived as conferring any extra benefits. The most immediate health issue concerns the risks and benefits of neonatal vitamin K prophylaxis which stem from two lines of evidence. One of these showing that without vitamin K prophylaxis, babies have a small but real risk of dying from being permanently brain damaged by vitamin K deficiency in the first six months of life. The other less certain evidence stems from a reported epidemiological association between intra-muscular vitamin K prophylaxis and the later development of childhood cancer in Bristol area of Great Britain (5). However, this association was not confirmed by subsequent studies carried out in Sweden (6) and the United States (7).

Currently an intramuscular injection of 0.5- 1 mg vitamin $K_1$ is administered to newborn babies at the time of birth as a prophylactic drug (8, 9). This protocol has been practiced in many countries and proved to be useful in the prevention of HDN. *In vitro* incubation of RBCs from G6PD deficient neonates to vitamin $K_1$ (Konakion) revealed that G6PD deficient RBCs were not at increased risk of oxidative damage from vitamin $K_1$ (10). Comparative studies carried out with menadione (vitamin $K_3$) and Konakion ($K_1$) showed that therapeutic doses of menadione caused oxidative stress in neonatal RBCs, whereas vitamin $K_1$ failed to do so (11). Assuming that administration of vitamin $K_1$ can cause a surge in vitamin K in some newborn babies, particularly healthy neonates, *in vivo* experiments have been carried out in weanling rats treated with different doses of vitamin $K_1$ in order to study possible changes in the rate of plasma lipid and protein oxidation. Antioxidant ability of plasma as judged by ferric reducing ability of plasma (FRAP assay) has also been studied to find out the responses of overall antioxidant activity against hepatotoxic effects of vitamin $K_1$. Data obtained from experiments

with growing animals have been compared with those of adult rats treated with vitamin $K_1$ throughout this study.

# MATERIALS AND METHODS

### Chemicals

Thiobarbituric acid (TBA), butylated hydroxyl-toluene (BHT) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were the products of Sigma chemical Co. (St. Louis, Mo.). Vitamin $K_1$ (Phylloquinone) was the product of Hoffmann-La Roche, Basel, Switzerland. 2, 4-dinitrophenyl hydrazine (DNPH), guanidium chloride, Iron (III) chloride.6H2O and menadione were purchased from E. Merck, Germany. All other reagents and solvents used were of analytical grade.

### Animals and treatment

Male Wistar rats were used in this study. At the time of experiment, adult animals were 3-4 months of age, weighing $220\pm 15$ g and weanling rats were $10\pm 2$ days old weighing $15 \pm 2$ g. Animals were purchased from Razi Research Institute (Karaj, Iran) and maintained in our animal house facilities. They were maintained on a commercial stock diet and water *ad libitum.*

Rats in each age-group were divided into 4 sub-groups. Vitamin $K_1$ was dissolved in DMSO and administered to groups of adult and growing rats. Various concentrations of vitamin $K_1$ solutions were prepared in DMSO and each experimental group received a final dose of 28, 56 or 84 mg/kg b.w. Each dose was divided into 3 equal injections and given i.p for 3 successive days. This protocol was used in order to maintain animals on a highest possible dose. Both adult and weanling control rats received vehicle alone i.e., DMSO. Animals were then anesthetized slightly with diethyl ether and blood was collected from the heart and transferred to tubes containing citrate buffer. Plasma was separated immediately and stored at 70˚C for further use.

Plasma lipid peroxidation was measured spectroflourimetrically using TBA reagent according to the method described by Brown and Kelly (12). The concentration of thiobarbituric acid reacting substances (TBARS) in unknown samples was calculated by plotting a standard curve of fluorescence (Excitation at 515 nm and emission at 553 nm) against MDA concentration obtained from the tetramethoxy propane (TMP) standards.

The carbonyl groups content of proteins was used as an index of protein oxidative damage. Plasma

protein carbonyl concentration was measured according to the dinitrophenyl hydrazine (DNPH) derivatization method described by Evans et al., (13). Briefly; proteins dissolved in 6% SDS were mixed with an equal volume of the DNPH solution and incubated at room temperature. Derivatized proteins were precipitated with TCA and the pellet was dissolved in 6 M guanidine. Absorbance was recorded at 370 nm for 45 min. Total plasma antioxidant capacity assay was performed using TPTZ reagent. This method measures the ability of the antioxidants contained in the sample to reduce ferric-tripiridyltriazine ($Fe^{3+}$-TPTZ) to a ferrous form ($Fe^{2+}$) which absorbs light at 593 nm. The FRAP levels of plasma were calculated by plotting a standard curve of absorbance against µM/L concentration of Fe (II) standard solution (14). Protein concentration of samples was measured by the method of Bradford (15) using bovine serum albumin as standard. For histological studies experiment groups of adult and weanling rats were treated i.p. with a dose of 56 mg/kg b.w of vitamin $K_1$ (dissolved in DMSO) which was given over 3 consecutive days. Corresponding control groups received equal volume of DMSO. All the animals were sacrificed 24 h after the last injection. Small portions of livers from the central lobes of adult and suckling rats were placed into 10% freshly prepared formalin. The tissues were allowed to fix for 48 h before being dehydrated, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin for histopathological observations.

### Statistical analysis

All the samples and standards were run in duplicate and the results were presented as mean±SEM. Statistical comparison between the experimental groups was performed by non-parametric Wilcoxon and Kruskal-Wallis tests using SPSS computer program. Statistical significance ($p<0.05$) between and within groups was determined by means of the Fischer method of multiple comparisons.

## RESULTS

### Biochemical studies

The levels of plasma thiobarbituric acid reacting substances (TBARS) in DMSO-treated (control) adult and growing rats were in the same range (Fig.1). These parameters remained unchanged in adult and growing rats treated with a low dose vitamin $K_1$, i.e., 28 mg/kg. However administration of higher doses of vitamin $K_1$ i.e., 56 or 84 mg/kg resulted in significant changes in the levels of plasma lipid peroxide formation in both the age-groups. Vitamin $K_1$ at a dose of 84 mg/Kg resulted in approximately 36% increase in plasma TBARS in adult rats. In contrast, there was about 26% decrease in plasma lipid peroxidation in young animals treated with the same dose of vitamin $K_1$. Protein carbonyls may be considered as a sensitive biomarker for protein damage. The level of protein carbonyls in DMSO-treated animals (controls) was found to be higher ($p<0.05$) in adults plasma as compared to that of weanling animals (Fig. 2). In addition, there was a slight decrease in protein carbonyls in plasma of adult rats treated with vitamin $K_1$, whereas vitamin $K_1$ failed to alter plasma carbonyl group levels in plasma of immature animals (Fig.2).
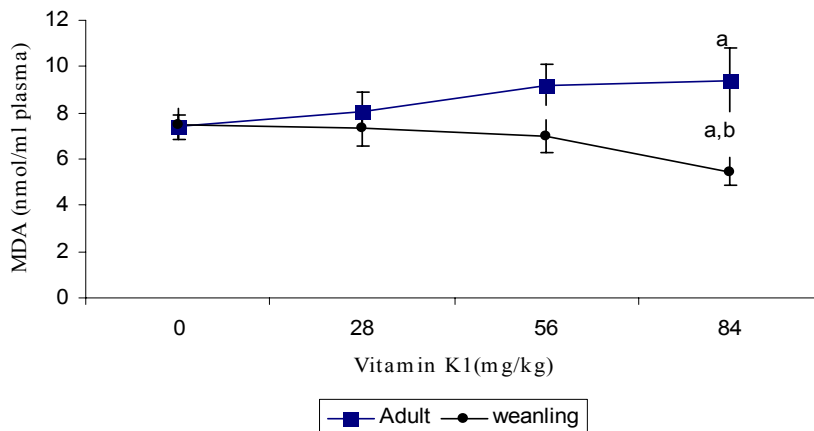


**Fig. 1.** Plasma concentration of thiobarbituric acid reactive substances (TBARS) in adult and weanling rats treated with vitamin $K_1$. Values are Mean ± SEM obtained of 4 analyses carried out in duplicate. **(a)** Significantly different from the respective control group. **(b)** Significantly different from the other age-group (P< 0.05)
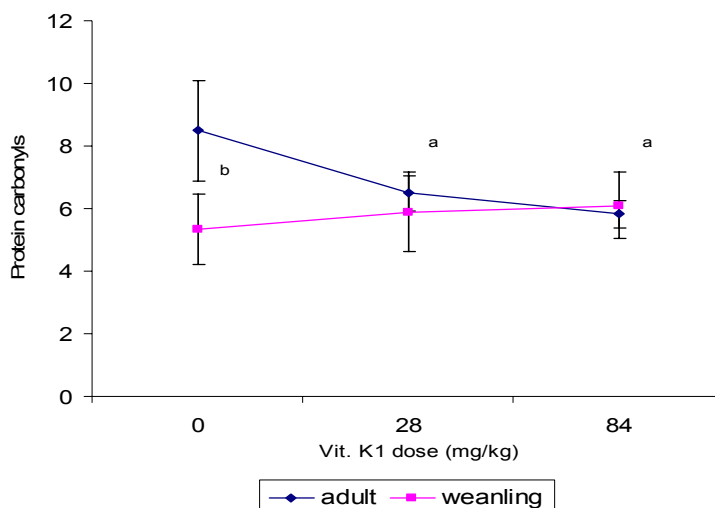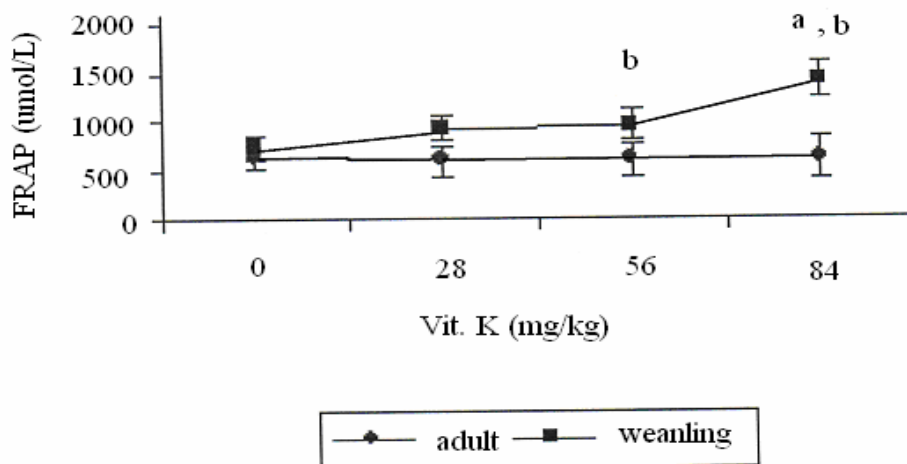
**Fig. 2.** Plasma protein carbonyls in adult and weanling rats treated with vitamin K1. Each point represents the mean of 4 duplicate analysis carried out on 4 separate tissues. Data are in terms of n mol /mg protein. **(a)** Significantly different from the respective control group. **(b)** Significantly different from the other age-group (P< 0.05)

Dose-dependent effects of vitamin $K_1$ on the total plasma antioxidant capacity as judged by ferric reducing ability of plasma assay (FRAP) of the two age-groups is illustrated in figure 3. Basically, FRAP activity was in the same range in samples obtained from adult and growing rats treated with DMSO (control groups). It is interesting to note that vitamin $K_1$ administration to immature rats resulted in a significant (P<0.05) increase in FRAP levels as compared to the corresponding control group. A dose of 28, 56 and 84 mg/kg b.w of vitamin $K_1$ resulted in 25, 41 and 92% increase in FRAP in immature rats. However under these circumstances vitamin $K_1$ failed to increase FRAP levels in adult animals treated with vitamin $K_1$.

**Histological observations:** Histological studies carried out on adult and weanling rats treated with overdose (56 mg/ kg b.w) vitamin $K_1$ 24 h prior to sacrifice and sampling, resulted in differential morphological changes in liver tissues. Under these conditions vitamin $K_1$ caused liver cell death in adult rats (Fig. 4A and 4 B). Whereas, the end point of toxic effect of vitamin $K_1$ in liver of immature rats was extensive accumulation of lipid droplets in the cells particularly in the mid-zonal region (Fig. 5A and 5B). Other morphological changes including focal edema and dilatation of sinusoids were common in liver preparations from both control (DMSO-treated) and vitamin $K_1$- treated animals.



**Fig. 3.** Effects of vitamin $K_1$ on total antioxidant ability of plasma (FRAP) in adult and weanling rats. Each point represents the mean of 4 duplicate analysis carried out on 4 separate tissues. **(a)** Significantly different from the respective control group. **(b)** Significantly different from the other age-group (P< 0.05)

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**A**                                                                 **B**
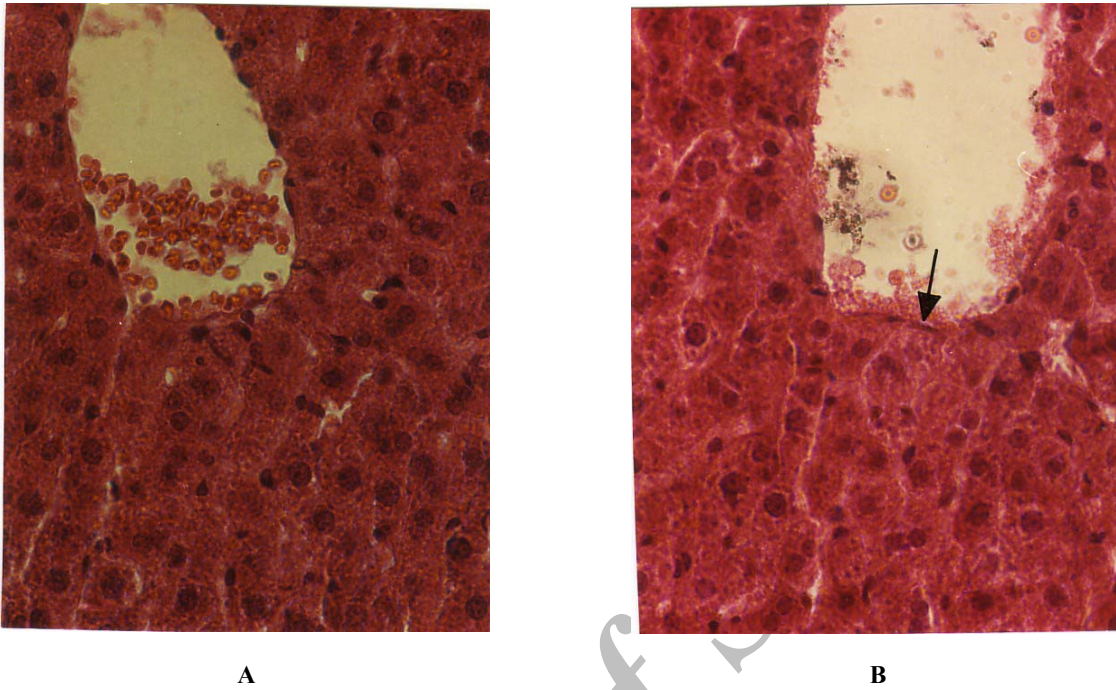
**Fig. 4.** Magnified view of liver sections of adult rats treated with vitamin $K_1$. **(A)** Control animals injected with DMSO only. The final volume of DMSO was 2.1 ml given for 3 days (700 µl/day) **(B)** Treated rats given 84 mg/kg b.w of vitamin $K_1$ dissolved in 2.1 ml of DMSO injected in 3 consecutive days and sacrificed 24 h after the last injection. Single arrow shows a dead hepatocyte



**A**                                                                 **B**
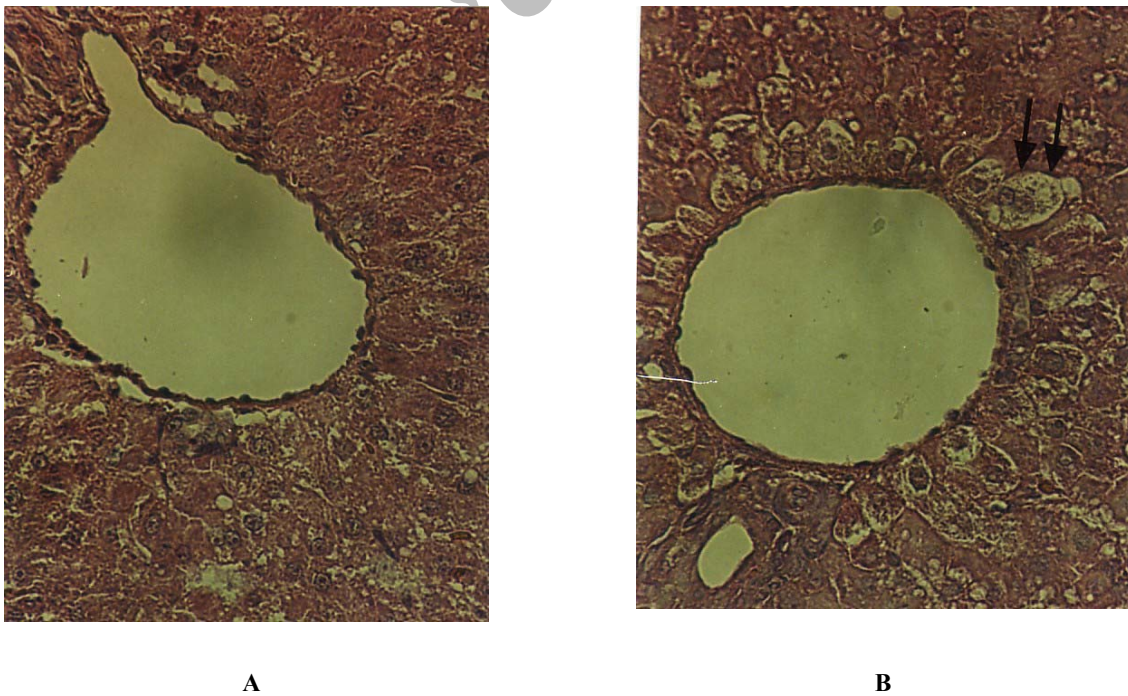
**Fig. 5.** Sections showing hepatic tissues of weanling rats. **(A)** Control group given DMSO only. The final volume of DMSO was 0.15 ml for 3 days (50 µl/day) **(B)** Treated young rats were given 84 mg/kg b.w of vitamin $K_1$ dissolved in DMSO (50 µl/day). Double arrow shows accumulation of fat droplets in hepatocytes

# DISCUSSION

Despite the routine practice of vitamin K$_1$ administration to neonates, there is a controversy over the administration of vitamin K$_1$ to all the neonates (16). Vitamin K$_1$ administration to premature neonates has been proved to be useful in the prevention of postnatal bleeding. However, due to inter-individual differences in vitamin K levels at birth, a surge in vitamin levels in some of the neonates particularly healthy neonates may occur. Selected plasma factors such as lipid peroxide (LP) products and protein carbonyls can be used as indices of oxidative damage. The plasma levels of LP products i.e. thiobarbituric acid reacting substances (TBARS) alone may reflect the damages which occur to the cell membranes in hepatocytes and red blood cells (RBCs). Regardless of the sources of TBARS formed in plasma of normal adult and immature rats, it was observed that basically plasma TBARS were within the same range in adult and immature rats. It seems that only very high doses of vitamin K$_1$ (>28 mg/kg) are capable of increasing plasma LP products in both the age-groups. The concentration of TBARS was differentially affected in response to higher doses of vitamin K$_1$ i.e., 56-84 mg/kg (Fig. 1). An important observation in this study was the difference in the plasma levels of TBARS in adult and weanling rats treated with vitamin K1. This difference was in favor of young rats because there was a significant suppression (~26%) in plasma levels of LP products. On these bases, one can suggest that immature rats are relatively less susceptible to oxidative damage caused by high doses of vitamin K$_1$. But the question arises is as to what is the role of antioxidant factors in age-related differences in LP formation. In this connection measurement of FRAP which indicates the overall antioxidant capacity of plasma revealed that FRAP was readily induced in immature rats in response to vitamin K$_1$. It seems that, a gradual increase in antioxidant activity of plasma of young rats under vitamin K$_1$ treatment precede the formation of lipid peroxide products. We have also shown that the changes in plasma biochemical parameters are reflected in the histopathological observations carried out on liver samples. It was observed that immature rats treated with vitamin K$_1$ suffered from relatively lesser liver damages as compared to adults under similar conditions of treatment. The endpoint of vitamin K$_1$ toxicity in immature livers was the accumulation of fat droplets in hepatocytes whereas in adults a number of cell deaths were counted. It is believed that in humans, the main antioxidants contributing to FRAP are uric acid and ascorbic acid. Plasma proteins and low molecular weight compounds containing the SH-group such as glutathione have very low activity in FRAP assay (14). Because plasma uric acid concentration is relatively lower in rats than in humans, other antioxidants probably play a dominant role in this species. In addition to the aforementioned non-enzymatic factors, the antioxidative enzymes such as, superoxide dismutase, glutathione peroxidase, catalase and glutathione S-transferases (GSTs) play an important role in modulation of drug-induced oxidative damage. In case of vitamin K$_1$, such age-dependent differences can be specifically taken into account for enzymes such as DT-diaphorase, which is directly involved in protection against vitamin K-related toxic metabolites (17,18).

Vitamin K$_1$-dependent induction in FRAP activity in immature animals reported here is reminiscent of our previous study on the induction of hepatic cytosolic GSTs in young rats treated with high dose of paracetamol or aflatoxin B1 (19,20). The implication of such age-related differences in GST activities was observed in the rate of glutathione (GSH) conjugate formation of paracetamol in immature liver (19). Furthermore, the role of inducible GSTs in protection of immature liver against lipid peroxidation has been established (21). Overall results show that only very high doses of vitamin K$_1$ (>28 mg/kg) are capable of increasing circulating LP products in adult and weanling rats. Vitamin K$_1$ administration to young rats which enhances the antioxidant capacity of plasma in weanling rats is responsible for reducing the relative production of LP products. The data indicate that inducible antioxidants in growing rats are important and contributing factors in protecting liver against oxidative damages caused by high doses of vitamin K$_1$.

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