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Vitamin K_1 Increases Sister Chromatid Exchange *in Vitro* in Human Leukocytes and *in Vivo* in Fetal Sheep Cells: A Possible Role for "Vitamin K Deficiency" in the Fetus

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ABSTRACT. The levels of the vitamin K-dependent clotting factors are markedly lower in the human fetus and newborn than in older infants and adults. Direct measurement of vitamin K_1 in cord plasma records low or undetectable levels. This phenomenon, although the norm, is referred to as vitamin K deficiency and is a significant risk factor for hemorrhage in the fetus and newborn. Sister chromatid exchange (SCE), which may be used as an index of mutagenic activity, was assayed in cultured leukocytes of placental and adult blood following phytohemagglutinin stimulation. The mean number of SCEs per metaphase in human placental blood was 3.32 ± SE 0.219 as compared with levels of 5.13 ± SE 0.273 in young adults ($p < 0.01$), and in the presence of added vitamin K_1 at a concentration of 1×10^{-6} M the SCE increased significantly in both adult and placental cells. *In vitro* SCE dose response curves to K_1 in the blood of fetal and maternal sheep were obtained. When five fetal sheep were given 1 mg of K_1 by catheter into the femoral vein the SCE increased from 3.94 ± SE 0.15 preinjection to 5.38 ± SE 0.23 at 24 h postinjection ($p < 0.01$). In the pretreatment fetal sheep, serum vitamin K_1 was below detectable levels in all seven animals in which it was assayed and reached levels as high as 0.3 $\times 10^{-6}$ M 1 h post-K_1 injection. The low level of K_1 in the fetus may in fact confer some biological advantage by reducing the risk of mutagenic events during a period of rapid cell proliferation. (*Pediatr Res* 22: 405–408, 1987)

Abbreviations

SCE, sister chromatic exchange
BP, benzo(a)pyrene
MC, mitomycin C
MFO, mixed function oxidase
PHA, phytohemagglutinin
PT, prothrombin time
BrdV, bromodeoxymidine
HPLC, high-performance liquid chromatography

It would seem an anachronism that the newborn is either vitamin K deficient or in precarious vitamin K balance at birth. The low levels of the K-dependent clotting factors place the newborn at some hemorrhagic risk. When bleeding does occur it responds to the injection of vitamin K, and the coagulation factors rapidly approach adult levels. Direct measurement of K_1 confirms the low or undetectable levels in cord plasma, and the large concentration gradient between mother and fetus suggests that K_1 does not readily cross the placenta (1). The phenomenon of fetal and neonatal K deficiency is not confined to man or other mammals but also occurs in birds. In North America it is a standard practice to supplement the diet of newly hatched chicks with menadione (vitamin K_3) as it is in most newborn nurseries to give parenteral K_1 to newborns. Why are fetus and newborn placed at hemorrhagic risk—has nature made a mistake or does K deficiency confer some advantage during fetal life and early infancy in man and other species? Recent observations that vitamin K_1 accelerates BP tumorogenesis in mice (2) and that diet-induced K_1 deficiency in mice inhibits hepatic BP metabolite-DNA adduct formation *in vivo* (3) suggest that high or even "normal" levels of K_1 may amplify potentially mutagenic or carcinogenic events. If so, higher levels would place the fetus and newborn at particular risk because of their high rates of cell proliferation.

To examine the problem more directly we studied the effect of vitamin K_1 on SCE in leukocytes from human adult and cord blood. In addition, the response to the *in vivo* administration of K_1, as monitored by SCE in leukocytes of fetal sheep, was determined.

METHODS

SCE in leukocytes was measured in the peripheral blood of 10 women between the ages of 21 and 43 and in blood obtained from the surface vessels of the placenta at the time of delivery or cesarean section of 10 normal full-term infants. We also assayed leukocytes from five fetal sheep between 130 and 135 days of gestation (full-term at 143 to 147 days) that weighed between 3.5 and 3.7 kg. Twenty to 24 h before beginning the study a catheter was placed in the femoral vein to allow sampling and injection. Blood was obtained for SCE and PT. One mg of vitamin K_1 (Konakion, Hoffman-La Roche) was injected and the sampling repeated 24 h later.

To measure the number of SCEs in human cells, lymphocytes were separated on a Ficoll-Hypaque gradient and 1×10^6 lymphocytes were added to 5 ml of medium. Because of the small volumes available from the fetal sheep, whole blood was used and 0.4 ml was added to 5 ml of medium. The culture system was RPMI medium 1640 (GIBCO Labs) with added penicillin, streptomycin, and neomycin to which was added 12% by volume of fetal bovine serum (GIBCO Labs). This culture system contained no vitamin K but for the minor component present in fetal bovine serum. To this culture 0.1 ml of PHA (Wellcome) was added at zero time and incubated at 37° C in 5% CO_2. At

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24 h BrdU, 6 μg/ml, was added together with one of the following: 1) vitamin K_1 (2-methyl-3phytyl-1,4 naphthoquinone) (Sigma); 2) BP (Aldrich); 3) MC (Bristol); 4) acetone 6 μl, which was the solvent for all test compounds (control). Following another 48 h of incubation, Colcemid (Sigma), 0.05 μg/ml of medium, was added and incubation continued for an additional 2 h. The lymphocytes were carefully protected from light during the entire incubation period. The cultures were centrifuged at low speed, the supernatant decanted and the red cells lysed in 0.075 M KCl. The leukocytes were gently mixed and washed three times in methanol: glacial acetic acid (3:1), refrigerated overnight at 4° C and slides prepared with Hoechst 33258 and Giemsa according to the method of Perry and Wolff (4). Twenty-five metaphase spreads were examined in each sample by a single technician who was unaware of the prior treatment of each specimen.

The serum concentration of vitamin K_1 was assayed by high-performance liquid chromatography on silica columns according to the method of Haroon and Hauschka (5) following the serum extraction procedure of Ueno and Suttie (6). The lower limit of vitamin K_1 detectable in serum was 0.5 ng/ml. Prothrombin times were done on plasma from fetal sheep using human brain thromboplastin as the thromboplastic agent.

RESULTS

The mean numbers of SCEs in the separated lymphocytes from human adult female and from fetal placental blood are shown in Tables 1 and 2. In Table 1 (adult) the *in vitro* incubation of lymphocytes with K_1, BP, and MC all produced a significant increase in SCE ($p < 0.01$) as compared with the controls. Both BP and MC produced significantly higher levels of SCE than did K_1 ($p < 0.01$). No difference was observed when K_1 was added together with either BP or MC as compared with those agents alone. Similar findings and levels of significance were observed for placental blood (Table 2). In comparing adult (Table 1) with placental values (Table 2) there is a significantly lower level of SCE in placental blood (control) ($p < 0.01$) and in placental cells incubated in the presence of K_1 ($p < 0.01$). No significant differences were noted between the placental and adult values in the cells exposed to BP or MC.

The vitamin K-deficient status of the sheep fetus was established by PTs varying from 25 to 43 s which, 24 h after K_1, had shortened to 16 to 23 s. The PTs of the dams were between 14 and 16 s. Figure 1 shows the *in vitro* dose response curve of sheep cells obtained at a single time point from a dam and her fetus. The SCE at a concentration of vitamin K_1 of 1×10^{-10} M was not different from the solvent control. As in the human the level of SCE is lower in the fetus. Both sets of observations increase with increasing concentrations of vitamin K_1 to a level of 1×10^{-6} M. In analyzing the data from all individual mitoses at each concentration interval the increase observed at 1×10^{-9} M as compared with 1×10^{-10} M in the fetus is significant ($p < 0.01$). No significant increase in the dam occurred until a K_1 level of 1×10^{-8} M had been reached.

Table 3 documents the SCE in five fetal sheep before and 24 h following the injection of 1 mg of vitamin K_1. In each fetus the SCE was greater following K_1 and, in the group considered as a whole, the mean preinjection level was 3.94 ± SE 0.15 as

Table 1. *Mean number of exchanges per metaphase in cultured lymphocytes from young women**

Subject	Control	K_1	BP	MC	K_1 + BP	K_1 + MC
1	3.9	7.4	16.5	18.3	16.5	18.3
2	4.2	6.0	16.9	18.9	16.7	17.2
3	5.7	8.5	14.7	18.8	14.4	
4	5.8	9.1	18.5	23.9	19.5	23.6
5	5.0	8.6	17.0	24.1	17.3	20.1
6	6.9	8.6	18.0	24.8	15.7	20.0
7	5.0	7.6	17.0	20.1	17.4	20.4
8	5.2	8.1	16.4	18.8	16.1	18.6
9	5.1	8.0	17.3	26.0	17.1	21.2
10	4.5	6.2	15.4	19.0	15.8	17.2
Mean	5.13	7.81	16.77	21.27	16.65	19.62
±SE	0.273	0.326	0.354	0.960	0.426	0.685

* Concentrations are: K_1, 1×10^{-6} M; BP, 1×10^{-6} M; MC, 1×10^{-8} M. Comparisons of control vs treatment groups were done by Dunnetts many to one test (24). Multiple comparisons between treatment groups were done by Bonferroni matched pair analysis (25).

Table 2. *Mean number of exchanges per metaphase in cultured lymphocytes from placental blood**

Placenta	Control	K_1	BP	MC	K_1 + BP	K_1 + MC
1	2.4	4.7	14.6	17.2	14.5	15.7
2	2.5	5.0	17.5	19.3	16.0	18.4
3	2.9	5.2	12.2	14.6	13.4	14.6
4	3.4	5.1	16.3	17.2	15.7	16.8
5	3.0	7.4	16.7	18.5	15.3	
6	3.8	6.6	16.8	20.7	16.4	16.4
7	4.2	6.6	17.5	19.7	17.6	17.8
8	3.0	5.3	15.1	15.2	14.3	14.7
9	3.5	5.2	11.5	13.0		
10	4.5	6.5	16.8	20.6	14.3	20.5
Mean	3.32	5.76	15.49	17.60	15.28	16.86
±SE	0.219	0.291	0.676	0.836	0.431	0.705

* Concentrations are: K_1, 1×10^{-6} M; BP, 1×10^{-6} M; MC, 1×10^{-8} M. Comparisons of control vs treatment groups were done by Dunnetts many to one test (24). Multiple comparisons between treatment groups were done by Bonferroni matched pair analysis (25).

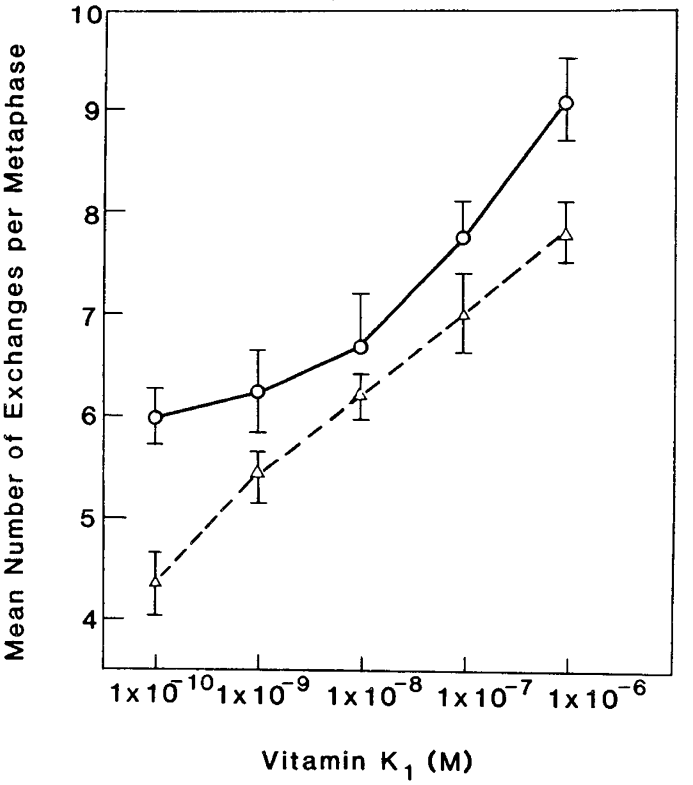

Fig. 1. Dose response curves (mean and SE of 25 observations per point) to increasing concentrations of K_1 on leukocyte SCE in one dam (O–O) and her fetus (Δ–Δ). The Kruskal-Wallis test (26) and the Wilcoxon rank sum test (26) were applied in making comparisons between the concentrations of vitamin K_1.

Table 3. *Mean number of exchanges per metaphase in fetal sheep leukocytes and serum K_1 levels before and 24 h after injection of 1 mg of K_1**

Fetus		SCE	K_1 (ng/ml)
1	Pre K_1	3.7	ND†
	Post K_1	5.8	9.1
2	Pre K_1	3.5	ND
	Post K_1	4.7	7.1
3	Pre K_1	4.2	ND
	Post K_1	5.0	9.6
4	Pre K_1	4.3	ND
	Post K_1	5.9	10.6
5	Pre K_1	4.0	ND
	Post K_1	5.5	24.8
Mean	Pre	3.94 ± 0.15	ND
±SE	Post	5.38 ± 0.23	12.24 ± 3.19

* Significance pre versus post $p < 0.01$. Paired t tests using Bonferroni t statistics (25) are applied to test for pre versus post differences in each group.

† Not detectable.

compared with 5.38 ± SE 0.23 24 h following K_1 ($p < 0.01$). When cells obtained preinjection were incubated *in vitro* with K_1 (1×10^{-6} M) the SCE increased to 6.0 ± SE 0.62 as compared to the control of 3.94 ± SE 0.15 ($p < 0.01$). However, there was no further increase found on *in vitro* incubation with K_1 (1×10^{-6} M) of postinjection cells.

Table 3 also records the levels of serum K_1 assayed before and 24 h after the injection of 1 mg of K_1. In no fetus was K_1 detectable in the preinjection sample. Twenty-four h after the injection of 1 mg of K_1 the levels ranged from 7.1 to 24. 8 ng/ml. In two fetal sheep in which sera were assayed before and 1 h after the intravenous injection of 1 mg of K_1 the levels were nondetectable preinjection and were 142.7 and 57.2 ng/ml 1 h after injection.

DISCUSSION

In 1969 the following appeared as an editorial footnote: "We believe that the weight of evidence—clearly indicates the benefits to be derived from the routine administration at the time of delivery of a small dose of vitamin K to all newborn infants. There seems to be no justification whatsoever for withholding this preparation and we would earnestly hope that the subject will not be reopened for at least another 10 years. Twenty years would be even better" (7). Although this practice is now almost routine in North America the basis of this regimen is still questioned elsewhere (8). The controversy arises in part from the fact that we are intervening in a natural phenomenon. Why the normal newborn reaches the outside world in a state requiring immediate supplementation with an essential vitamin has not been explained.

That the vitamin K-dependent coagulation factors are low in the fetus and newborn and that bleeding may occur and is corrected or prevented by vitamin K is firmly established. Hemorrhagic disease of the newborn on this basis may occur within the first few days of life or, in solely or mainly breast-fed babies, as late as 4 to 8 wk after birth. Direct measurement of vitamin K_1 in plasma by HPLC records levels in fasting adults from 0.10 to 0.66 ng/ml but K_1 was not detectable by this method in cord plasma (1). Although the low levels of K, either measured directly or inferred by the low levels of the K-dependent clotting factors, is the norm in the human fetus and in other species, it is common to refer to this state as "vitamin K deficiency." Is it not possible that this level of vitamin K provides an advantage to the embryo or fetus which compensates for the neonatal risk? The present findings suggest that these lower levels of K may in fact confer some advantage on the rapidly growing fetus.

SCE represents the interchange of DNA at homologous chromosomal loci and is dependent on both chromosomal breakage and repair. Although there is not perfect agreement between the ability of a compound to produce SCE and its mutagenicity or carcinogenicity there is a high degree of correlation (9). Some known carcinogens such as diethylstibesterol are detected by SCE although found to be negative in the Salmonella assay (10, 11). Although vitamin K_1 was not mutagenic in Salmonella strains TA98, TA100, or TA2637 (12), the demonstration that K_1 supplementation increased SCE at a concentration of 1×10^{-6} M *in vitro* and at a dose of 1 mg *in vivo* must be considered a significant chromosomal event.

In the present studies of human leukocytes the level of SCE was significantly lower in cord cells than in those of normal adults (Tables 1 and 2). A higher frequency of SCE in adults than in children (ages 0.2 to 4.5 yr) has been observed and was postulated to be on the basis of continuing exposure to environmental mutagenic agents (13). SCE in fetal cells was found to be less than in maternal marrow cells in C57BL/6J mice prior to mutagenic exposure (14). In both adult and fetal leukocytes, *in vitro* incubation with K_1 at a concentration of 1×10^{-6} M induced a significant increase in exchanges although much less than the classical mutagens BP and MC. That the *in vitro* effect of K_1 was not additive to that of BP and MC may indicate that the exchanges induced by K_1 are occurring at some of the same chromosomal sites as those produced by BP and MC. Exchange is not a random event and certain chromosomal regions are preferential sites of high exchange frequency (15).

Figure 1 shows the SCE dose response curves to K_1 in the leukocytes of a dam and her fetus. As in the human the control values (K_1 at 1×10^{-10} M) in the fetus are less than in the mother. Both maternal and fetal SCE levels increase with increasing concentration of K_1 but the fetus responds significantly at lower concentrations of K_1. This may relate to the higher concentration of K_1 in maternal plasma so that the effect of the addition of K_1 at concentrations below 1×10^{-8} M is diluted out by the K_1 normally present. The *in vivo* injection of 1 mg of K_1 to the fetus produces a significant increase in SCE. This dose of K_1 is equivalent to that recommended for full-term human newborns of comparable weight.

The vitamin K_1 level in each fetus was below the level of detection of the assay (<0.5 ng/ml). In all fetal sheep K_1 was easily measurable 24 h after the intravenous injection of 1 mg of K_1 and ranged from 7.1 to 24.8 ng/ml. In two other fetal sheep in which serum K_1 was assayed 1 h postinjection these levels were 142.7 and 57.2 ng/ml. These levels are well above those required to produce SCE in fetal sheep cells *in vitro* (Fig. 1).

It is significant that in the human adult the total body pool, excluding ingested and bacterial vitamin K in the gut, is extremely small. Bjornsson *et al.* (16) estimated the turnover time of intravenously administered vitamin K_1 to be 153 min and the body pool to be replaced every 2.5 h. Duello and Matschiner's (17) data suggest the hepatic pool, which is the primary site of K_1 storage, to be about 300 μg. Although multiple forms of menaquinones (K_2) are also present in the hepatic pool (17) it is not an uncommon clinical observation in patients deprived of oral intake and placed on broad-spectrum antibiotics that the vitamin K-dependent clotting factors frequently fall within 6 to 7 days which, when the half-lives of the factors are considered, indicates that K depletion occurs within 72 to 96 h. This has been documented in a series of case reports (18, 19).

In addition to the direct chromosomal effects demonstrated in this study, it has previously been shown, in a microsomal system, that low concentrations of K_1 may increase the MFO metabolism of the procarcinogen BP to more proximate carcinogenic metabolites (3). The MFO system mediates the metabolism of a number of hydrophobic organic molecules including the polycylic hydrocarbons such as BP. The initial (phase I) reaction produces primary and secondary metabolites which are frequently more toxic or mutagenic than the parent compound. These are then

conjugated (phase II) with glutathione, glucuronic acid, or sulfate prior to excretion (20). In the vitamin K-deficient chick embryo system, the *in vivo* administration of K_1 augments BP metabolism (phase I) with a concomitant decrease in at least one phase II pathway suggesting that K_1 could assume an adjuvant role in mutagenicity in this embryo system (21). If K_1 at levels marginally above those present *in vivo* modulates MFO metabolism to enhance production of potentially toxic, mutagenic, or carcinogenic metabolites, then maintaining K_1 at a cellular concentration only sufficient to meet the immediate needs of the γ-carboxylase system and without cellular excess is a biological advantage. This is particularly so in the fetus which, although it has an active and inducible MFO system (22), has a much reduced capacity for the conjugation and excretion of such metabolic products. Conjugation with glucuronic acid is one such pathway and glucuronyl transferase activity is only 20% of the adult levels at birth although it rapidly increases to reach adult levels by 2 to 4 days postpartum (23).

These findings do not negate the use of therapeutic or prophylactic vitamin K_1 in the newborn. Hemorrhagic disease of the newborn may be fatal or leave the infant with devastating disability. However, it is necessary to reconsider the phenomenon of "vitamin K deficiency," which is the norm for this period of growth and development, in terms of the role it may play in the protection of the fetus *in utero.*

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