Analysis of Chromosome Aberrations and Sister Chromatid Exchanges in Peripheral Blood Lymphocytes of Newborns after Vitamin K Prophylaxis at Birth

MARLIES CORNELISSEN, DOMINIQUE SMEETS, GERARD MERKX, RONNEY DE ABREU,
LOUIS KOLLEE, AND LEO MONNENS

Departments of Pediatrics and Human Genetics, University Hospital Nijmegen, Nijmegen, The Netherlands

ABSTRACT. In many countries vitamin K prophylaxis at birth is recommended to prevent bleeding in infants due to vitamin K deficiency. Because the incidence of clinical vitamin K deficiency is very low, such a vitamin K administration should be completely safe. However, an increase in sister chromatid exchanges in lymphocytes of fetal sheep 24 h after injection of vitamin K₁ has been reported. Therefore, a study concerning genotoxicity of vitamin K₁ in man was conducted. Sister chromatid exchanges and chromosome aberrations were analyzed in peripheral blood lymphocytes of six newborns 24 h after intramuscular administration of 1 mg vitamin K₁ and in six control neonates. The mean number of sister chromatid exchanges per metaphase in the vitamin K group was 8.88 ± 1.22 as compared with 9.05 ± 1.14 in the control group (NS). The mean number of chromosome aberrations per 100 mitoses was 3.00 ± 2.61 in the vitamin K group and 2.50 ± 1.87 in the control group (NS). Vitamin K₁ plasma concentrations ranged from 115 to 1150 ng/mL (255 to 2555×10^{-9} M) in the supplemented group, a 5000-fold rise as compared with the control group ($p < 0.01$). We did not find any evidence for genetic toxicity due to the administration of 1 mg vitamin K₁ intramuscularly to the newborn child. (*Pediatr Res* 30: 550–553, 1991)

Abbreviations

SCE, sister chromatid exchanges
CA, chromosome aberrations
BrdU, bromodeoxyuridine

The problem of bleeding in infants due to vitamin K deficiency has been recognized worldwide. In many countries vitamin K prophylaxis at birth is recommended to prevent this hazard. Introduction of such a prophylaxis appeared to have spectacular results on the incidence of this so-called Haemorrhagic Disease of the Newborn (1). Because the incidence of clinical vitamin K deficiency is very low, this vitamin K supplementation should be completely safe. However, in 1987, Israels *et al.* (2) reported a dose-dependent increase of the frequency of SCE in lymphocytes after *in vitro* exposure to vitamin K₁. *In vivo* exposure, tested in five fetal sheep given 1 mg of vitamin K₁ i.v., also resulted in an increase in SCE 24 h after injection. SCE represent

interchanges of DNA at apparently homologous chromosomal sites after replication. These exchanges presumably involve DNA breakage and reunion. Although there is no perfect agreement between the ability of a compound to produce SCE and its mutagenicity or carcinogenicity, there is a high degree of correlation (3). Israels *et al.* (2) postulated that the relative vitamin K-deficient state of the newborn may in fact play a role in the protection of the fetus *in utero* by reducing the risk of mutagenic events during this period of rapid cell proliferation. The question arises whether the results of the study of Israels *et al.* indicate a genotoxic or carcinogenic risk to man, specifically when the vitamin is administered during early life. Moreover, Golding *et al.* (4) reported a statistical association between childhood cancer and vitamin K administration (among other drugs in the 1st wk of life). This association was found unexpectedly and fitted no prior hypothesis. The need for further research was stressed by the authors. To our knowledge, cytogenetic studies concerning genotoxicity of vitamin K₁ in man have as yet not been performed.

Apart from SCE assays, another way of testing for cytogenetic mutagenicity is to analyze CA. CA reflect damage of the chromosome- or chromatid-type and can be observed as breaks, acentric fragments, minutes, ring chromosomes, and gaps. Gaps are defined as regions in the chromatid structure that are achromatic, thus, the chromosome structure is not really discontinued (5). Because CA and SCE are two different cytogenetic end points of mutagenic action, they are complementary (6). Neither CA nor SCE have been studied before in human neonates exposed to vitamin K₁.

The aim of this case-controlled clinical trial is to determine whether the administration of 1 mg vitamin K₁ intramuscularly after birth influences the occurrence of SCE and CA in cultured peripheral blood lymphocytes.

MATERIALS AND METHODS

The study was approved by the local medical ethical committee. Informed consent was obtained from the parents. Twelve healthy breast-fed newborns, delivered in our hospital, were recruited for this study. Selection criteria were delivery at term (37–42 wk), birth weight over 2500 g, and Apgar score at 5 min of seven or more. The mother could not be on a special diet, smoke, abuse alcohol, or take any medication during the last month of pregnancy, except for iron or folic acid. Arterial cord blood pH, mode of delivery, and medication of the mother during labor were recorded. All infants had an arterial cord blood pH over 7.24, except for subject 7, who had a pH of 7.17. All children were delivered vaginally, except for subject 1, who was born by cesarean section. In two cases oxytocin was given to the mother during labor (subjects 3 and 11).

Received March 13, 1991; accepted July 5, 1991.

Correspondence and reprint requests: Marlies Cornelissen, M.D., Department of Pediatrics, University Hospital Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands.

Supported by the Dutch "Praeventiefonds."

The infants were randomly divided into two groups; six received 1 mg vitamin K₁ (Konakion; Hoffman-La Roche, Basel, Switzerland) intramuscularly, the other six received no medication. Some characteristics of the neonates are given in Table 1. No item differed significantly between the groups. After 24 h, 5 mL of blood were drawn by aseptic venipuncture: 2 mL of heparinized blood and approximately 3 mL of blood with no additive. The latter was kept in the dark immediately after sampling, centrifuged (3000 × *g* for 5 min), and stored at -20°C until vitamin K₁ determination. All samples were coded to provide blind cytogenetic analysis.

Vitamin K₁. Vitamin K₁ was measured in 1-mL serum samples by a two-step HPLC procedure, according to the method of Lambert *et al.* (7, 8). A few modifications were applied. The assays were performed with two Spectra Physics SP8800 systems (Spectra Physics, Santa Clara, CA) equipped with Rheodyne 7125 manual injectors (Rheodyne, Cotati, CA), with 100-μL sample loops. Vitamin K₁₍₂₅₎ was used as an internal standard. The first HPLC step was used for preseparation of vitamin K₁ and K₁₍₂₅₎ on a microspher-Si column (Chrompack, Middelburg, The Netherlands). The locations of vitamin K₁ and internal standard were detected with a variable wavelength detector model 770 (Spectra Physics) set at 248 nm. Final separation and quantitation were performed during the second HPLC step, using a microspher-C18 column (Chrompack) for separation and postcolumn chemical reduction with tetramethylammonium-octahydridotriborate for fluorescence detection (λ_{ex} = 325 nm, λ_{em} = 450 nm) with a Waters 470 fluorescence detector (Waters Associates, Millipore Corp., Milford, MA). Concentrations were calculated from relative peak heights of vitamin K₁ versus known amounts of internal standard. The lower limit of detection was 0.045 ng/mL; however, the detection limit was dependent on the recovery of internal standard and the amount of serum extracted.

Cytogenetics. Peripheral lymphocyte cultures were initiated within 2 h after blood collection. For each culture two containers with medium (8 mL RPMI 1640 medium, supplemented with 15% FCS, heparin, phytohemagglutinin, penicillin, and streptomycin) were each supplied with 0.2 mL neonatal whole blood. From all infants two cultures were set up: one with and the other without BrdU (final concentration 10 μg/mL). BrdU was present in the medium during the whole culture period. All cultures were grown in the dark at 37°C for exactly 72 h. Colcemid (final concentration 0.2 μg/mL) was added 90 min before harvesting. Chromosome spreads were made according to routine procedures. The standard cultures without BrdU were applied for aberration screening by one technician. From every child 100 mitoses were studied after conventional Giemsa staining and all CA were recorded and photographed. The cultures with BrdU were used for SCE studies. From every infant 25 complete mitoses were photographed in fluorescence microscopy after the chromosomes had been stained with acridine orange (9). In this way the number of SCE could easily be counted from the photograph.

For statistical evaluation the unpaired *t* test was applied for study-population characteristics, SCE, and CA results. Vitamin K₁ concentrations were compared by Wilcoxon's two-sample test. All values are given as means ± SD.

Table 1. Study population characteristics (mean ± SD)

	Vitamin K	Control
<i>n</i>	6	6
Male:female	2:4	3:3
Gestational age (wk)	39.5 ± 1.8	40.4 ± 0.7
Birth wt (g)	3338 ± 608	3817 ± 299
Apgar score at 5 min	8.8 ± 1.2	9.7 ± 0.5
Arterial cord blood pH	7.27 ± 0.03	7.29 ± 0.09
Age at venipuncture (h)	24.6 ± 0.4	27.8 ± 5.8

RESULTS

The vitamin K₁ concentrations and the number of SCE and CA of each subject are presented in Table 2. SCE results are missing for subjects 4 and 8 because of bad growth of BrdU cultures. Less than 20 mitoses were suitable for SCE analysis. Conventional growth was adequate and chromosome aberrations were counted. During determination of vitamin K₁ in subject 4, a technical error was encountered. Because of the small volumes of serum available from the neonates, the procedure could not be repeated.

Twenty-four h after injection of 1 mg vitamin K₁, plasma levels ranged from 114.6 to 1150.5 ng/mL (255 to 2555 × 10⁻⁹ M). Vitamin K₁ concentrations in the supplemented group were raised some 5000-fold as compared with the control group (*p* = 0.006). In two subjects of the control group, the vitamin K₁ concentration was not even detectable. Because of differences in volume of serum available, the detection limit ranged from 0.045 to 0.198 ng/mL in these samples.

In the vitamin K-supplemented group, the mean number of SCE was 8.88 ± 1.22 per metaphase compared with 9.05 ± 1.14 in the control group. The groups did not differ significantly (*p* = 0.82).

When the total number of chromosome and chromatid breaks as well as gaps was taken into account, a mean number of 3.00 ± 2.61 CA per 100 mitoses was detected in the vitamin K group. In the control group a mean number of 2.50 ± 1.87 per 100 mitoses was found (NS, *p* = 0.71). When gaps were excluded, the mean numbers amounted to 1.50 ± 1.05 and 2.50 ± 1.87, respectively. Still, no significant difference was present (*p* = 0.28).

DISCUSSION

As would be expected, 24 h after the intramuscular administration of 1 mg vitamin K₁ serum concentrations vitamin K₁ were extremely high, the mean being 536 ng/mL. This finding is in accordance with the mean levels of 444 ng/mL reported by McNinch *et al.* (10) and of 348 ng/mL by Luccock *et al.* (11). McNinch *et al.* (10) detected a peak level of 1781 ng/mL after 12 h. This shows that the blood lymphocytes in the vitamin K group certainly have been exposed to extremely high levels of vitamin K before sampling. Previous studies have shown that the plasma level declines rapidly (12).

Several short-term mutagenicity test systems have been developed to identify agents that can adversely affect the genetic material of cells (The American Industrial Health Council, 13). This property may indicate the potential for a number of adverse effects, one of which is carcinogenicity. There is a clear and justifiable rationale for the use of genetic toxicology assays, but they are less than perfect. Accuracy, which is a combination of sensitivity and specificity, is about 60%. Therefore, one assay will be insufficient (13, 14). The Ames/*Salmonella typhimurium* assay is the test most commonly used for evaluation of chemicals *in vitro* (15). Vitamin K₁ was not mutagenic in this test (16). A negative Ames test, however, does not exclude carcinogenicity, inasmuch as a negative predictive value of only 51% was calculated (14). In other words, performance of an Ames test alone is inadequate to determine whether vitamin K₁ is genotoxic. Moreover, Israels *et al.* (2) did report an increase of SCE in lymphocytes after *in vitro* and *in vivo* exposure to vitamin K₁. *In vitro* exposure was analyzed in cultured lymphocytes of human placental blood and adult female blood with added vitamin K₁ at a concentration of 1 × 10⁻⁶ M (450 ng/mL). Consequently, the lymphocytes that were stimulated to replicate by phytohemagglutinin were exposed for at least 48 h to a high concentration of vitamin K₁. This is quite different from *in vivo* situations, where high concentrations of vitamin K₁ are only temporary and lymphocytes normally do not divide. *In vivo* exposure was tested in fetal sheep *in utero* given 1 mg of vitamin K₁ i.v. SCE were

Table 2. Number of SCE and CA in metaphases of cultured lymphocytes of newborns who did or did not receive 1 mg vitamin K₁ intramuscularly after birth

Infant morbidity after birth							
Subject	SCE			CA*			Vitamin K (ng/mL)
	n†	Mean	SD	Chrbr	Chrmatbr	Gap	
Vitamin K							
1	25	9.96	2.92	0	1	0	114.6
2	24	7.96	2.46	0	0	0	203.9
3	26	8.03	3.13	0	2	5	545.0
5	20	10.45	3.68	0	3	0	443.4
8				0	1	4	1150.5
11	24	8.00	2.67	2	0	0	757.4
Mean	23.8	8.88	2.97	0.33	1.17	1.5	535.8
Control							
4				0	2	0	
6	20	10.30	2.92	0	6	0	0.188
7	21	8.52	2.69	0	1	0	<0.045
9	24	7.80	2.77	1	2	0	0.130
10	29	8.41	2.10	1	0	0	0.132
12	25	10.24	3.20	0	2	0	<0.198
Mean	23.8	9.05	2.74	0.33	2.17	0	

* Per subject, 100 mitoses were screened. Chrbr, number of chromosome breaks; Chmatbr, number of chromatide breaks.

† Number of cells investigated for SCE.

measured before and 24 h after injection. Each fetus showed an increased number of SCE after vitamin K₁.

Because the aim of the present investigation was to determine whether there are indications that vitamin K may have genotoxic effects in the human neonate, we opted for an *in vivo* study in humans with the intention to reflect the clinical situation, especially because several unknown factors may hamper generalization of *in vitro* and animal studies to clinical practice. SCE and CA analyses were performed on cultured lymphocytes after exposure of the neonate *in vivo*. Accordingly, the vitamin will be subjected to the spectrum of activating and deactivating enzymes as far as these are developed in the newborn (17). Detoxification or formation of toxic degradation products might be different in fetal lambs as compared with human neonates. In fetal mice SCE response was found to decline with gestational age (18). Administration of mitomycin C, a mutagen that does not require metabolic activation, and of cyclophosphamid, a drug activated by the liver into a potent alkylating agent, both resulted in a decline in SCE with gestational age. Repair capacities may vary with gestational age.

Fortunately, no carcinogenic metabolites are reported for vitamin K₁. In the liver vitamin K is first reduced to vitamin K hydroquinone and subsequently oxidized to vitamin K-2,3-epoxide. This oxidation is connected with the carboxylation reaction in which glutamic acid residues of vitamin K-dependent coagulation factors are carboxylated. Subsequently, vitamin K-2,3-epoxide is converted back into natural vitamin K and the cycle is complete. In adults vitamin K₁ is excreted in urine and bile as more polar metabolites (carboxylic acids) conjugated with glucuronic acid. An enterohepatic circulation is present (19). After supplementation with vitamin K₁, urinary and fecal excretion is virtually complete after 3 d and amounts to about 60% of the dose (19). Whether fetal vitamin K metabolism differs from adult metabolism is unknown but conceivable.

Besides advantages, *in vivo* testing carries the problem that unknown confounding factors may disturb the conclusions. Age, sex, tobacco smoking, medication, and alcohol abuse may be confounders (20). In the present study these items were excluded or otherwise randomized. We were unable to detect an increase in SCE 24 h after the administration of 1 mg vitamin K₁ intramuscularly.

Because additional negative results were achieved in the CA analysis, which is a less sensitive but more specific short-term mutagenicity test (14), a genotoxic effect is improbable.

When the effects of supplementation with vitamin K are

considered, not only plasma concentrations (and lymphocyte exposure) have to be taken into account. Concentrations in other tissues may differ. It might be possible that because of accumulation hepatic concentrations higher than plasma concentrations are reached. The hepatic vitamin K₁ level in unsupplemented term neonates is reported to be about 1 ng/g liver (fresh weight) with a wide range (21). This is 10-fold higher than the plasma level of about 0.1 ng/mL in our control group. Shearer *et al.* (21) have reported hepatic vitamin K₁ concentrations 10 h after intramuscular administration of 0.5 to 1.0 mg vitamin K₁ of approximately 200 ng/g and after 1 to 4 d, 500–2000 ng/g. These levels are comparable to the plasma levels in our supplemented group, so there are no indications that higher concentrations are reached in the liver than in the blood after a single intramuscular dose of vitamin K₁.

In conclusion, as yet there is no evidence for cytogenetic toxicity due to the administration of 1 mg vitamin K₁ intramuscularly to the newborn infant.

REFERENCES

1. Matsuzaka T, Yoshinaga M, Tsuji Y, Yasunaga A, Mori K 1989 Incidence and causes of intracranial hemorrhage in infancy: a prospective surveillance study after vitamin K prophylaxis. *Brain Dev* 11:384–388
2. Israels LG, Friesen E, Jansen AH, Israels ED 1987 Vitamin K₁ 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. *Pediatr Res* 22:405–408
3. Latt SA, Schreck RR, Lovedays KS, Dougherty CP, Shuler CF 1980 Sister chromatid exchanges. *Adv Hum Genet* 10:267–331
4. Golding J, Paterson M, Kinlen LJ 1990 Factors associated with childhood cancer in a national cohort study. *Br J Cancer* 62:304–308
5. Buckton KE, Evans HJ 1973 Methods for the Analysis of Human Chromosome Aberrations. World Health Organization, Geneva
6. Gebhart E 1981 Sister chromatid exchange (SCE) and structural chromosome aberration in mutagenicity testing. *Hum Genet* 58:235–254
7. Lambert WE, De Leenheer AP, Lefevre MF 1986 Determination of vitamin K in serum using HPLC with post-column reaction and fluorescence detection. *J Chromatogr Sci* 24:76–79
8. Lambert WE, De Leenheer AP 1987 Simplified post-column reduction and fluorescence detection for the high-performance liquid chromatographic determination of vitamin K_{1(20)}}. *Anal Chim Acta* 196:247–250
9. Verma RS, Lubs HA 1975 A simple R banding technic. *Am J Hum Genet* 27:110–117
10. McNinch AW, Upton C, Samuels M, Shearer MJ, McCarthy P, Tripp JH, L'E Orme R 1985 Plasma concentrations after oral or intramuscular vitamin K₁ in neonates. *Arch Dis Child* 60:814–818
11. Lucok M, Hartley R, Wild NJ 1987 HPLC determination of vitamin K₁ in neonatal plasma following oral or parenteral supplementation with Konakion. *J Liquid Chromatogr* 9:191–203
12. Widdershoven J, Lambert W, Motohara K, Monnens L, De Leenheer A, Matsuda I, Endo F 1988 Plasma concentrations of vitamin K₁ and PIVKA-

- II in bottle-fed and breast-fed infants with and without vitamin K prophylaxis at birth. *Eur J Pediatr* 148:139-142
13. Kier LD 1988 Comments and perspective on the EPA workshop on "the relationship between short-term test information and carcinogenicity." *Environ Mol Mutagen* 11:147-157
  14. Tennant RW, Margolin BH, Shelby MD, Zeiger E, Haseman JK, Spalding J, Caspary W, Resnick M, Stasiewicz S, Anderson B, Minor R 1987 Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science* 236:933-941
  15. Ames BN, McCann J, Yamasaki E 1975 Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat Res* 31:347-364
  16. Tikkanen L, Matsushima T, Natori S, Yoshihira K 1983 Mutagenicity of natural naphthoquinones and benzoquinones in the Salmonella/microsome test. *Mutat Res* 124:25-34
  17. Perry PE, Thomson EJ 1984 The methodology of sister chromatid exchanges. In: Kilbey BJ, Legator M, Nichols W, Ramel C (eds) *Handbook of Mutagenicity Test Procedures*. Elsevier, New York, pp 495-529
  18. Kram D, Bynum GD, Senula GC, Bickings CK, Schneider EL 1980 *In utero* analysis of sister chromatid exchange: alterations in susceptibility to mutagenic damage as a function of fetal cell type and gestational age. *Proc Natl Acad Sci USA* 77:4784-4787
  19. Shearer MJ, McBurney A, Barkhan P 1974 Studies on the absorption and metabolism of phyloquinone (vitamin K₁) in man. *Vitam Horm* 32:513-542
  20. Wulf HC 1990 Monitoring of genotoxic exposure of humans by the sister chromatid exchange test. Methodology and confounding factors. *Dan Med Bull* 37:132-143
  21. Shearer MJ, McCarthy PT, Crampton OE, Mattock MB 1988 The assessment of human vitamin K status from tissue measurements. In: Suttie JW (ed) *Current Advances in Vitamin K Research*. A Steenbock Symposium. Elsevier, New York, pp 437-452

Announcement

American Board of Nutrition

1992 Examination

The next certifying examination of the American Board of Nutrition will be administered on May 1 and 2, 1992 in conjunction with the Annual Meeting of The American Society for Clinical Nutrition in Baltimore, MD.

Individuals with a doctoral degree (M.D., Ph.D., or equivalent) and appropriate nutrition background are eligible to take the examination. M.D. candidates must be certified by a primary board recognized by the American Board of Medical Specialties and have completed at least 2 years of appropriate nutrition training at the time of the examination.

Deadline for receipt of academic records and sponsoring letters is January 2, 1992.

For more detailed information, write to: American Board of Nutrition, 9650 Rockville Pike, Bethesda, MD 20814. Telephone: (301) 530-7110.