The Influence of Dietary Fatty Acids and Vitamin E on Plasma Prostanoids and Liver Microsomal Alkane Production in Broiler Chickens with Regard to Nutritional Encephalomalacia

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Summary Nutritional encephalomalacia (NE) in broiler chicken is considered as a peroxidative dysfunction caused by vitamin E-deficient diets. A feeding experiment was performed to investigate the consequences of feeding different fats in combination with increasing amounts of vitamin E on liver lipid peroxidation and plasma prostanoid pattern. Newly hatched chicks from hens on a vitamin E-poor diet were fed with either mainly linolenic, linoleic or oleic acid-rich oils in a vitamin E-deficient (5 ppm) basic diet. The animals were supplemented with vitamin E on three levels (0, 20 or 120 ppm). On appearance of the first symptoms of NE after 8 days post-hatching, the animals were examined. Typical symptoms with a high incidence only occurred in the group fed linoleic acid and 5 ppm vitamin E. Plasma prostanoids and microsomal alkane production in liver as a measure of endogenous lipid peroxidation were determined. The dietary conditions affected plasma prostaglandin E2 and thromboxane A2, but not prostacyclin. However, it seems unlikely that the prostanoids are involved in the pathogenesis of NE. Liver lipid peroxidation increased in vitamin E deficiency. The level of alkanes depended on the type of fat supplied. The consequences of the different dietary fats in combination with vitamin E deficiency on peroxidative metabolism of broiler chickens are evident, indicating that a high level of oxidative stress is imposed by the linoleic acid-rich fat.

Key Words lipid peroxidation, vitamin E, ethane, pentane, prostanoids, encephalomalacia, fatty acids, chicken

The pathogenesis of nutritional encephalomalacia (NE) in broiler chicken is not completely understood, although NE is obviously caused by a peroxidative dysfunction. The disease occurs two to three weeks post-hatching when linoleic acid-rich diets low in vitamin E are fed (1, 2). Histological alterations reported earlier are swelling of the endothelia and microthrombosis in the cerebellum. It is
presumed that the plasma prostanoids play a role in such microcirculatory disturbances. Experiments on this subject have been published, however the results are conflicting (3, 4). Nutritional aspects of eicosanoids are recognized now, as reviewed recently (5).

Alkanes have been used to estimate the peroxidative status of a tissue under examination (6). Oxidative attack on n-6 and n-3 polyunsaturated fatty acids leads to the formation of the volatile hydrocarbons (7). Alkanes are formed through Fe-catalysed reaction of the lipid hydroperoxides to alkoxy radicals. Thermal decomposition via β-scission (8) produces the pentyl or ethyl radical, which then abstracts an H-atom of a neighbouring fatty acid to form the hydrocarbons pentane or ethane.

In early age chicken tissue, phospholipids are easily manipulated by the dietary fat (9). For both prostanoid synthesis and alkane production, membrane polyunsaturated fatty acids are the substrates. Therefore, it is expected that these parameters are changed by dietary conditions. This study reports the consequences of feeding different fats in combination with increasing amounts of vitamin E on peroxidative metabolism of broiler chickens, as evaluated by the microsomal alkane production in liver. Furthermore, the influence of dietary conditions on the pattern of plasma prostanoids with regard to the development of NE is investigated.

**EXPERIMENTAL**

To obtain day-old chicks regularly low in tissue α-tocopherol, chickens (Lohmann Broiler Meat, not sexed) were bred from hens marginally supplemented with vitamin E (10 ppm). From the 30th week, the eggs were collected for 8±2 days and incubated for 3 weeks. The newly hatched chicks were kept under permanent light and 32°C room temperature with 60% humidity. For 2 days, a vitamin E-deficient (5 ppm α-tocopherol) basic diet (Table 1) without fat or additional vitamin E was given. Afterwards, the animals were randomly assigned to 9 groups of 8–10 chicks each kept in a wire battery. The environment in the fully air-conditioned room was kept constant by maintaining the temperature (±1°C) at 32°C during the first week and by reducing it weekly by 2 to 3°C until 25°C. Humidity was maintained at 60±5%, and light was provided for 24 h. Each group got the basal purified diet supplemented with three levels of vitamin E (0, 20 or 120 ppm) and 10% fat (Table 1). The composition of the different dietary fats and the resulting fatty acid patterns are listed in Table 2. The feed was mixed every week again and stored in the dark at −22°C. The peroxide value was <1 millequivalents of peroxide per kg feed and did not increase during storage.

The first symptoms of NE appeared at day 8. On each of the following 3 days, one-third of the animals were randomly selected for examination every day. The chicks were anaesthetized by i.m. injection of 75 mg Metomidat-HCl/kg (Hypnodil™, Janssen, Germany), and 3 to 5 ml blood was obtained by cardiac puncture. Ten mg EDTA and 10 µg indomethacin per ml blood were added to inhibit
Table 1. Composition of diets.

| Basic diet                        | g/kg |
|-----------------------------------|------|
| Corn starch                       | 392.0|
| Soya protein (concentrated)       | 328.0|
| Mineral mix*                      | 70.0 |
| Cellulose                         | 40.0 |
| Vitamin mix**                     | 11.0 |
| Phenylalanine                     | 3.2  |
| DL-Methionine                     | 2.5  |
| Glycine                           | 2.0  |
| Cholinchloride                    | 1.3  |

Supplements

| Fat (linolenic, linoleic or oleic acid-rich) | 100.0 |
| Vitamin E premix (0, 20, 120 ppm)          | 50.0  |

*Composition of the mineral mix (g/kg): CaHPO₄·2H₂O, 19.6; CaCO₃, 20.5; KH₂PO₄, 15.2; NaHCO₃, 9.60; MnSO₄·H₂O, 0.38; FeSO₄·7H₂O, 0.54; MgSO₄·3H₂O, 0.38; KIO₃, 0.01; CuSO₄·5H₂O, 0.036; ZnCO₃, 0.16; CoCl₂·6H₂O, 0.0034; NaMoO₄·2H₂O, 0.009; NiCl₂·6H₂O, 0.13; Na₂SeO₃·5H₂O, 0.000333. **Composition of the vitamin mix: Vitamin A, 10,000 IU; Vitamin D₃, 2,000 IU; Vitamin B₃-nitrate, 2 mg; Vitamin B₂, 6 mg; Ca-D-Pantothenate, 12 mg; Nicotinic acid, 40 mg; Folic acid, 1 mg; Vitamin B₆-HCl, 4 mg; Vitamin K₃, 2 mg; Vitamin B₁₂, 0.02 mg.

Table 2. Composition (g/kg) and fatty acid pattern (weight %) of the dietary fats.

| Dietary fat         | Source                          | g/kg | % n-3 | % n-6 | % n-9 | % sat. |
|---------------------|---------------------------------|------|-------|-------|-------|-------|
| Linolenic acid-rich | Linolenic acid 62% (Serva, Heidelberg) + Tallow | 86   | 56    | 14    | 18    | 12    |
|                     | Linoleic acid-rich Soya triglyceride, re-esterified | 100  | 5     | 55    | 22    | 15    |
| Oleic acid-rich     | Lard, vit. E-stripped (ICN, Meckenheim) + Oleic acid 82% (Merck, Darmstadt) + Linoleic acid 62% | 67   | 6     | 7     | 58    | 23    |

Cyclooxygenase activity. Afterwards, the animals were decapitated, completely bled and the liver was excised. Centrifugation of the blood samples with 2,000 × g for 15 min resulted in a platelet-poor plasma. The supernatants were assayed for prostanoids with commercial kits available from Amersham Buchler, Braunschweig. By radioimmunoassay with magnetic separation, prostacyclin was determined as 6-keto-prostaglandin F₁₅g and prostaglandin E₂ (PGE₂) after derivatisation with methyloximate-HCl. Thromboxane A₂ was measured as thromboxane B₂ with a scintillation proximity assay.

Alkane production was measured according to the method of Fuhrmann et al.
(10) with some modifications. Five g of liver were homogenised in a Potter-Elvehejm homogenizer with 5 mM TRIS-maleate buffer (pH 7.4) and 0.15 mM KCl with four strokes at 1,000 rpm. The homogenate was centrifuged for 10 min at 1,400 × g at 4°C. The supernatant was then centrifuged at 25,000 × g for 45 min at 4°C. The microsomal cell sap was saved and centrifuged for 60 min at 4°C at 104,000 × g. The microsomal pellet was resuspended in TRIS-maleate-KCl buffer and an aliquot directly used for measurement of alkane production.

For stimulation of peroxidation, 0.5 ml of the sample was pipetted to a 6.7-ml gas-tight vial with a Teflon-coated screw cap. Then, 0.5 ml of a TRIS-maleate-KCl buffer (TRIS-maleate 100 mM + KCl 0.15 mM, pH 7.4), 0.1 ml ADP-Fe³⁺ solution (ADP 32.8 mM, Fe³⁺ 1.64 mM) and 0.1 ml NADPH+H⁺ 1.22 mM was added followed by incubation for 30 min in a shaking water bath at 37°C. One ml of the headspace was taken and analysed by gas chromatography. The chromatographic conditions were as follows: gas chromatograph, Packard-Becker Model 419, Delft, The Netherlands; oven temperature, 60°C for 7 min, elevated to 90°C at 4°C/min; injector temperature, 130°C; detector temperature, 130°C; gas flow, N₂, 8 ml/min; column, 5 m steel, diameter 2 mm, filled with Porasil C (0.15–0.20 mm) from Serva, Heidelberg, Germany. The results were documented with a Varian integrator using an external alkane standard containing ethane and pentane (Messer-Griesheim, Duesseldorf).

Statistical calculations were done with the UNISTAT statistical software (Vers. 3.0, 1994; Unistat Ltd., London, England). Data obtained were tested by two-way analysis of variance with the factors vitamin E and dietary fat. Differences between the harmonic group means were determined using the 95% Student Neuman-Keuls interval. Data for plasma thromboxane were not normally distributed and therefore were transformed logarithmically for statistical analysis.

RESULTS

The incidence of NE started at day 8 after hatching. The disease only occurred in the group fed linoleic acid without vitamin E supplementation. Microsomal alkane production in liver and plasma prostanoids were determined during the following 3 days. Prostacyclin (range 12.4–23.7 pg/100 ml plasma; data not shown) was not influenced by vitamin E and dietary fat. PGE₂ (Fig. 1) was significantly decreased by feeding linolenic acid in comparison to oleic acid. Linoleic acid feeding was intermediate. Vitamin E had no significant effect on this parameter. Thromboxane A₂ values (Fig. 2) were influenced by dietary fat and vitamin E. Feeding linolenic acid leads to a significant decrease in comparison to linoleic acid. However, the latter leads to significantly lower thromboxane values in vitamin E deficiency.

The liver alkane production (C₂+C₅, Fig. 3) in the groups supplemented with 125 ppm vitamin E was very low, and differences between the dietary fats were not observed. Alkane production increased significantly when 25 ppm and 5 ppm were
Fig. 1. Plasma prostaglandin E₂ (determined as methyl-oximated prostaglandin E₂) in response to dietary fat ($p=0.0041$) and vitamin E (n.s.). Each column represents the group $M±SD$ ($n=9$). A, B denotes significant differences due to dietary fats (95% Student-Newman-Keuls interval).

Fig. 2. Plasma thromboxane A₂ (determined as thromboxane B₂) in response to dietary fat ($p=0.0023$) and vitamin E ($p=0.0084$). There is a significant interaction between both factors ($p=0.0364$). Each column represents the group $M±SD$ ($n=5$). Group means not sharing common letters (a, b) are significantly different (95% Student-Newman-Keuls interval).
Fig. 3. Sum (C2+C5) of ethane (C2) and pentane (C5) production from liver microsomes in response to dietary fat (C2+C5: p<0.0001, C2: p=0.0016, C5: p<0.0001) and vitamin E (C2+C5: p<0.0001, C2: p<0.0001, C5: p<0.0001). There is a significant interaction between both factors on C2+C5 (p<0.0001) and C5 (p<0.0001). The columns and numbers represent the group M±SD (n =9). Group means not sharing common letters (a, b, c, d) are significantly different (95% Student-Newman-Keuls interval).

|                | 5 ppm | 25 ppm | 125 ppm vitamin E |
|----------------|-------|--------|-------------------|
| **C2**         | 6.04 a| 4.78 ab| 0.87 d           |
| ±1.7           | ±2.0  | ±1.0   |                   |
| **C5**         | 1.33 c| 1.15 c | 0.15 d           |
| ±0.3           | ±0.4  | ±0.2   |                   |

Microsomal ethane production was the same in all groups supplemented with 125 ppm vitamin E. On the other hand, in the groups receiving 25 ppm vitamin E, significantly more ethane was produced in the animals fed n-3 fatty acids compared to the others. In the deficient groups there was a significantly lower ethane production in the oleic acid-fed groups compared to the others. Within the linoleic and oleic acid groups, ethane values of the deficient animals were significantly higher than those supplemented with 25 ppm vitamin E.

**DISCUSSION**

Based on the histological picture seen in NE, it has been postulated that thrombotic events are involved in the pathogenesis of the disease (4) although experimental evidence is sparse. The physiology of prostanoids in chicken has already been studied. In this species, prostacyclin is not produced by aortic tissue.
and has no anti-aggregative activity. The stable metabolite of plasma prostacyclin determined in our study is not affected by dietary fat and vitamin E. This is different from rats (12) where prostacyclin synthesis is depressed in vitamin E deficiency.

A number of studies have shown that PGE\textsubscript{2} synthesis in mice (13) and excretion in humans (14) are depressed by the addition of n-3 fatty acids to the diet. This has been found also in skeletal muscle (15) and aortal rings of the chicken (4). Our results show that plasma PGE\textsubscript{2} levels are affected in the same manner. It has been proved earlier that PGE\textsubscript{2} has an anti-aggregatory effect in chicken (16). However this is not consistent with the protective effect of n-3 fatty acids against NE observed in this and other studies (4).

From earlier studies, it is known that serum thromboxane A\textsubscript{2} as the pro-aggregatory prostanoid in chicken is decreased in vitamin E deficiency (3). This was confirmed in our experiment when only linoleic acid was fed. The decrease in blood levels of thromboxane A\textsubscript{2} is probably not due to decreased synthesis, for the production is obviously not affected by vitamin E (4). All these results lead us to the conclusion that thromboxane A\textsubscript{2} is not a primary factor in the development of NE. In summary, plasma thromboxane A\textsubscript{2} is easily manipulated in broiler chicken by dietary fat and vitamin E. PGE\textsubscript{2} is affected only by feeding fatty acids. However, it is unlikely that prostanoids play an essential role in the pathogenesis of NE.

From the data on microsomal alkane production, it is obvious that the supplementation with 125 ppm vitamin E suppresses the \textit{in vitro} peroxidation of membrane fatty acids completely, confirming the results of an experiment wherein chickens were fed with different levels of linoleic acid (17). Vitamin E 25 ppm allows higher alkane production rates independent of the type of dietary fat used. Over all vitamin E levels, the ethane production arising from n-3 fatty acids was higher in the groups fed linolenic acid in comparison to those receiving linoleic or oleic acid, indicating an increased incorporation of n-3 fatty acids into liver membranes. This is supported by the liver phospholipid data (unpublished) of an own experiment with the same dietary fatty acids. According to those data, the ratio of polyunsaturated fatty acid families in liver phospholipids is 44 to 56 (n-6: n-3), when linolenic acid is fed. The ratio of pentane and ethane arising from n-6 and n-3 respectively in these groups is 17 to 83. The feeding of linoleic acid gives a ratio of 84 to 16 (n-6: n-3) in polyunsaturated fatty acids of liver phospholipids. However, in these groups, the ratio between pentane and ethane was roughly balanced (43 to 57). The results suggest that the distribution pattern of pentane and ethane shows limited dependency from the fatty acid pattern of the liver phospholipids. This problem already appeared earlier for pentane and n-6 fatty acids from chicken microsomal phospholipids (17). These results indicate that there is no linear relation between tissue pentane production and its n-6 fatty acid content. A disproportionate release of ethane and pentane was also observed in a hydrocarbon breath test with rats fed different fatty acids (18). Furthermore, the
pentane to ethane ratio determined here is different from an earlier study with rats fed linoleic acid rich corn oil (6) reporting a ratio of 75 to 25. However, in these trials, lipid peroxidation was induced by halogenated hydrocarbons.

Under vitamin E deficiency, there was no difference in ethane production between the linoleic and linolenic acid-rich groups referring to a comparable degradation of n-3 fatty acids in both groups. However, the pentane production originating from n-6 fatty acids was considerably higher in the deficient group fed linoleic acid. Comparable results have been obtained earlier in vitamin E-deficient rats fed corn oil or menhaden oil (19). TBA-reactive substances arising mainly from n-3 fatty acids were 1.38 times higher, when the rats received menhaden oil. However, hexanal as a secondary peroxidation product of n-6 fatty acids was 6.35 times higher in the rats fed corn oil in comparison to those fed menhaden oil.

In conclusion, the appearance of NE occurring in the vitamin E-depleted chicks fed linoleic acid goes along with a higher production of pentane not compensated for by lower ethane production. Presumably, the linoleic acid-rich fat imposes high levels of peroxidative stress. The protective effect of linolenic acid (1), although in principle more susceptible to oxidation, is conceivable via the enrichment of membranes with n-3 at the expense of n-6 fatty acids. Under such a condition, the production of pentane and maybe other decomposition products of n-6 fatty acids are decreased, so that signs of NE are prevented.

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REFERENCES

1) Budowski, P., Leighfield, M. J., and Crawford, M. A. (1987): Nutritional encephalomalacia in the chick: An exposure of the vulnerable period for cerebellar development and the possible need for both omega-3 and omega-6 fatty acids. Br. J. Nutr., 58, 511–520.
2) Sallmann, H.-P., Fuhrmann, H., Molnar, S., and Stegmanns, T. (1991): Endogenous lipid peroxidation in broiler chickens under dietary loads. Fat. Sci. Technol., 93, 457–462.
3) Bruckner, G., Infante, J., Combs, G. F. Jr., and Kinsella, J. E. (1983): Effects of vitamin E and aspirin on the incidence of encephalomalacia, fatty acid status and serum thromboxane levels in chicks. J. Nutr., 113, 1885–1890.
4) Vericel, E., Budowski, P., and Crawford, M. A. (1991): Chick nutritional encephalomalacia and prostanoid formation. J. Nutr., 121, 966–969.
5) Sardesai, V. M. (1992): Biochemical and nutritional aspects of eicosanoids. J. Nutr. Biochem., 3, 562–579.
6) Gavino, V. C., Dillard, J. C., and Tappel, A. L. (1984): Release of ethane and pentane from rat tissue slices: Effect of vitamin E, halogenated hydrocarbons, and iron overload. Arch. Biochem. Biophys., 233, 741–747.
7) Lawrence, G., Cohen, G., and Machlin, J. (1982): Ethane exhalation by vitamin E deficient rats. Ann. N.Y. Acad. Sci., 393, 227–228.
8) Sevanian, A., and Hochstein, P. (1985): Mechanism and consequences of lipid peroxidation in biological systems. *Ann. Rev. Nutr.*, 5, 365–390.

9) Anderson, G. J. (1994): Developmental sensitivity of the brain to dietary n-3 fatty acids. *J. Lipid Res.*, 35, 105–111.

10) Fuhrmann, H., Balthazary, S. T., and Sallmann, H.-P. (1994): Bioefficiency of different tocopherols in chicken as assessed by haemolysis test and microsomal pentane production. *Br. J. Nutr.*, 71, 605–614.

11) Claey, M., Wechsung, E., Herman, A. G., and Nugteren, D. H. (1981): Lack of prostacyclin biosynthesis by aortic tissue of the chicken. *Prostaglandins*, 21, 739–749.

12) Meydani, M., Meydani, S. N., and Blumberg, J. E. (1993): Modulation by dietary vitamin E and selenium of clotting whole blood thromboxane A2 and aortic prostacyclin synthesis in rats. *J. Nutr. Biochem.*, 4, 322–326.

13) Meydani, M., Meydani, S. N., Shapiro, A. C., Macauley, J. B., and Blumberg, J. E. (1991): Influence of dietary fat, vitamin E, ethoxyquin and indomethacin on the synthesis of prostaglandin E2 in brain regions of mice. *J. Nutr.*, 121, 438–444.

14) Ferretti, A., Judd, J. T., Ballard-Barbash, R., Nair, P. P., Taylor, P. R., and Clevidence, B. A. (1991): Effect of fish oil supplementation on the excretion of the major metabolite of prostaglandin E in healthy male subjects. *Lipids*, 26, 500–503.

15) Olomu, J. M., and Baracos, V. E. (1991): Prostaglandin synthesis and fatty acid composition of phospholipids and triglycerides in skeletal muscle of chicks fed combinations of flaxseed oil and animal tallow. *Lipids*, 26, 743–749.

16) Bult, H., Wechsung, E., Houvenaghel, A., and Herman, A. G. (1981): Prostanoids and hemostasis in chickens: Anti-aggregating activity of prostaglandins E1 and E2, but not of prostacyclin and prostaglandin D2. *Prostaglandins*, 21, 1045–1058.

17) Klaus, A.-M., Fuhrmann, H., and Sallmann, H.-P. (1995): Peroxidative and antioxidative metabolism of the broiler chicken as influenced by dietary linoleic acid and vitamin E. *Arch. Geflügelk.*, 59, 135–144.

18) Kivits, G. A. A., Ganguli-Swarttouw, M. A. C. R., and Christ, E. J. (1981): The composition of alkanes in exhaled air of rats as a result of lipid peroxidation in vivo: Effects of dietary fatty acids, vitamin E and selenium. *Biochim. Biophys. Acta*, 664, 559–570.

19) Hu, M.-L., Frankel, E. N., Leibovitz, B. E., and Tappel, A. L. (1989): Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. *J. Nutr.*, 119, 1574–1582.