Serum vitamin E concentration and osmotic fragility in female long distance runners

Running title: **Vitamin E and haemolysis in female athletes**

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Abstract

The objective of this study was to assess the nutritional adequacy in sub-elite runners with irregular menstrual function (IR, n=10) and in a comparable group of runners with regular menstrual function (R, n=10), with special focus on vitamin E. Based on three days' records of weighed dietary intake, mean energy intake of micro and macro nutrients were estimated. Both estimated daily energy intake (IR: 9.9 ± 0.4, R: 12.2 ± 0.7 MJ), (Mean ±SE), and intake of dietary fat, (IR:61 ± 6 g, R: 98 ± 12 g), were lower in athletes with irregular menstrual function than in athletes with regular menstrual function; $p = 0.01$. Estimated intake of vitamin E was below recommended levels in both groups. Serum levels of α-tocopherol were used to evaluate vitamin E status, and showed normal levels in athletes with regular menstrual function (27.3 ± 3.6 μmol/L), while athletes with irregular menstrual function showed sub-normal levels (15.7 ± 0.8 μmol/L); $p = 0.01$. Furthermore, post exercise osmotic fragility in red blood cells was inversely related to resting α-tocopherol levels.

The results indicate that irregular menstrual function in athletes on a low-fat diet is associated with low levels of circulating α-tocopherol, rendering the red blood cells more susceptible to haemolysis in connection with physical activity.

Introduction

Vitamin E is a generic term that refers to four different tocopherols and four different tocotrienols. Among the tocopherols, α-tocopherol is the most biologically potent isomer and the only isomer actively retained in the blood (DRI, National Academy of Sciences, 2000*)*. Body stores of alpha-tocopherol are mainly found in the liver and fat tissue, and alpha-tocopherol is also embedded in cell membranes, protecting the unsaturated fatty acids of phospholipids in the lipid bi-layers against damaging peroxidation (Machlin LJ., 1984).

Overt vitamin E deficiency with neurological abnormalities is rare in otherwise healthy subjects. Slight to moderate vitamin E deficiency is generally asymptomatic and therefore not readily identified (Yokota et al., 1990), although an increased tendency of haemolysis may be observed at serum α-tocopherol levels below (14-11.6 $\mu mol \cdot L^{-1}$) (Gontzea & Nicolau, 1973; Shaskey & Green, 2000). Even if other deficiency signs are unspecific, manifestations may occur following a reduced defence capacity against reactive oxygen species (ROS) (Bendich, 1988; Wang, Huang, & Chow, 1996). The antioxidant effect of Vitamin E is considered to play an important role in the protection against several diseases, including cancer (Heinonen et al., 1998) and cardiovascular disease (Stampfer et al., 1993; Stephens et al., 1996), and oxidized low-density lipoprotein seems to play a key role in the initiation and progression of atherosclerosis (Thomas, 2000).

We do not know if there is an association between low levels of α-tocopherol and reproductive failure observed as menstrual irregularities, but reproduction failure is a well known symptom of vitamin E deficiency in many animal species (Brigelius-Flohe et al., 2002).

Increasing evidence of low dietary intake of vitamin E in modern Western societies has been presented within the general population (Maras et al., 2004) as well as in female athletic groups (Mullins, Houtkooper, Howell, Going, & Brown, 2001;

Jonnalagadda, Bernadot, & Nelson, 1998; Ziegler, Nelson, & Jonnalagadda, 1999). Additionally, a low fat diet is suspected to reduce the absorption and bioavailability of vitamin E (Dimitrov et al., 1991; Roodenburg, Leenen, het Hof, Weststrate, & Tijburg, 2000; Wolf, 2007), making dietary estimates of Vitamin E an uncertain predictor for vitamin E status (Ford & Sowell, 1999). As a consequence, vitamin E status should be evaluated based on the plasma levels of α- tocopherol, especially when fat intake is expected to be low.

It has previously been reported that a low energy availability may be the one of the main reasons for menstrual irregularities (Loucks, 2003) and also that weight-stable irregularly menstruating athletes seem to have a slight energy deficit primarily associated with a low intake of fat (Tomten & Hostmark, 2006). To learn more about vitamin E status in female athletes, the main focus of this study was to estimate nutritional intake, measure serum α-tocopherol levels and susceptibility to haemolysis in female runners with and without regular menstrual function.

Material and method

Participants

Ten competitive sub-elite runners with irregular menstrual function (IR) and ten runners with similar race results and with regular menstrual function (R) participated in the study. The two groups had similar height, weight, body mass index, body fat mass, lean body mass and fat% (Table 1).
All participants were female Caucasians aged 17 to 40 yrs, free of injury and otherwise healthy (Table 1), on no current medication, including oral contraceptives. The fitness level, and amount and intensity of physical training were similar in the two groups (Table 2).

Table 1. Background variables (Mean ± SEM)

Group	N	Age (Yr)	Height (cm)	Weight (kg)	BMI ($kg \cdot m^{-2}$)	LBM* (kg)	Body fat (kg)	VO_2 $mL*(kg*min)^{-1}$
R	10	35±2	168±1	59±2	19.5±0.2	44.1±0.7	19.3±1.2	59.8± 1.9
IR	10	27±2	168±1	57±1	19.8±0.4	45.1±1.1	19.3±1.4	59.3± 1.4

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
BMI = Body mass index
LBM = Lean body mass
VO_2 = Maximal Oxygen Consumption

Table 2. Physical activity per week (Mean ± SEM)

Group	N	Amount of training Hours: min	Mean training intensity Pulse rate
R	10	6: 42 ± 3: 23	142 ±13
IR	10	7: 33 ± 2: 41 NS	137± 11 NS

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
NS= No significant difference between groups R and IR

The haemoglobin concentration was normal in both groups (R=14.7±0.3 $g \cdot dL^{-1}$ and IR=14.6 ±0.5 $g \cdot dL^{-1}$). According to a pre test interview on nutritional behaviour, the participants were in general conscious of their nutritional intake but considered their food intake to be normal, and none of them used potentially pathogenic weight control regimes. There were no vegetarians in either group. The group of athletes with menstrual dysfunction consisted of runners who were defined as amenorrheic (n=7), i.e. with missing menstrual bleeding for at least 3 consecutive months during the last year, and oligomenorrheic (n=3), i.e. with missing menstrual bleeding during the previous year, for time periods that were shorter than 3 consecutive months. The hypo-estrogenic status of the runners with irregular menstrual function was verified by measurements of serum estradiol from blood (Table 3). None of these runners had menstrual bleeding one month prior to, or during the investigation.

The regularly menstruating runners were considered eumenorrheic with menstrual bleeding every 28 to 35 days. Serum content of estradiol measured during the follicular phase, showed estradiol levels within the normal range (Table 3).

Table 3. Hormones (Mean ± SEM)

Group	N	E2 ($nmol \cdot L^{-1}$)	LH ($IU \cdot L^{-1}$)	FSH ($IU \cdot L^{-1}$)	TSH ($nmol \cdot L^{-1}$)	f-T4 ($pmol \cdot L^{-1}$)
R	9	0.32±0.08*	7.6±1.4*	5.8±0.7†	1.9±0.4	13.9±0.5*
IR	10	0.10±0.01	2.8±1.0	4.0±0.5	2.9±1.3	11.9±1.6

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
* $p<0.01$ between groups R and IR
† $p<0.05$ between groups R and IR
E2 = estradiol
LH =Luteinizing Hormone
FSH = Follicle stimulating hormone
TSH =Thyrotropin – Thyroid stimulating hormone
f-T4 =Free thyroxine

Methods

The study was approved by the local ethics review committee and the Data Inspectorate of Norway.

Food intake

Food registration took place during the month of June, at the start of the competitive summer season.

The participants weighed and recorded all their intake of food and drinks using an electronic scale on two weekdays, and one weekend day (Saturday or Sunday). Peelings and left-overs were deducted. The athletes were instructed not to record food intake on competition days or days close to a competition.

Emphasis was put on the importance of unrestrained intake. The participants were asked to register all snacks and sweets which they sometimes ate "off the record". The athletes were told that their record would be used only as part of a group registration. They were further informed that if they should happen to record intake on a day when the meals were more generous than usual (parties, celebrations), they would probably be counter-balanced by other runners in the group, whose intakes would be below the usual amount, due to normal fluctuations.

The dietary data were analyzed using the Norwegian Nutritional Computer Analysis Program (1991) which is based on the Norwegian Food Composition Tables (1990). When special recipes were supplied by the participants, each ingredient was entered separately. Mean daily intakes were estimated to be one third of the individual total recorded amounts.

Serum analyses

The eumenorrheic runners had regular menstrual function. Serum concentration of estradiol was measured during the follicular phase, within 10 days after the start of a menstrual bleeding. Blood samples from the runners with irregular menstrual function were sampled at any time of the athletes' convenience.

To avoid inter-assay variation, all samples were analyzed in one batch.

Follicle stimulating Hormone (FSH), thyrotropin (TSH) and estradiol were analyzed using the Auto-DELFIA time resolved fluoroimmunoassay (Wallac Oy, Turku, Finland). For the follicle stimulating hormone the limit of quantization was 1 IU/L. None of the participants had results below the quantization limit.

Luteinizing hormone (LH) and free thyroxine (f-T4) were analyzed using the DELFIA time resolved fluroimmunoassay (Wallac Oy, Turku, Finland). For the luteinizing hormone, the limit of quantization was 0.6 IU/L. Four of the athletes with irregular menstrual function had results below the quantization limit and were given the value 0.5 IU/L. Serum concentrations cholesterol and triacylglycerol were determined enzymatically using Nycotest kits (Nycomed A/S, Oslo).

Determination of α-tocopherol

Previous reports have found discrepancies between estimated dietary intakes of vitamin E and serum levels of α-tocopherol (Ford et al., 1999; Guilland et al., 1989), and to verify any substantial differences in vitamin E intake, blood was drawn from 13 of the 20 participants. The remaining 7 athletes lived too far away. In order to evaluate vitamin E status, a resting blood sample was drawn, at least

two hours after a light meal, and the serum was analyzed for α-tocopherol. After solid phase (C-18 material) extraction in acetonitrile, α-tocopherol was isolated by high pressure liquid chromatography, using a Suplex pKb-100, C-18 column (250*4.6 mm). The fluorescence detector had an excitation wavelength of 294 nm, and an emission wavelength of 330 nm. The method was linear in the range 1-60 μmol. The coefficient of variation was < 3%.
The frozen samples were thawed and analyzed in one batch.
The ratio of α-tocopherol to the sum of triacylglycerol and cholesterol was calculated as suggested by Thurnham et al. (Thurnham, Davies, Crump, Situnayake, & Davis, 1986).

Osmotic fragility

In order to determine the resistance of red blood cells to haemolysis before and after a standardized exercise, 13 of the 20 participants, in addition to previous tests, also exercised for 20 min at the anaerobic threshold (HF =171±2 in athletes with regular menstrual function and HF= 172±3 in athletes with irregular menstrual function).

Blood was drawn from an indwelling catheter before and after exercise and 10 μl heparinised blood was added to vials containing 2 ml hypotonic sodium chloride (Dacie, 1974). Vials containing 0,45% concentrations of NaCl in Hepes buffer (pH 7.4) incubated at 37° C were used to examine osmotic fragility. After centrifugation for 5 min at 1000 rpm spectrophotometric readings (540 nm) were made. Results are given as percent of maximal haemolysis using 0 % NaCl solution (100% haemolysis) and 0,9% NaCl solution (Blank) as references.

Body composition

Fat and lean body mass measurements were performed using dual X-ray absorptiometry (Lunar DPX-1, Lunar Inc., Madison Wisconsin). The coefficient of variation was estimated using 12 participants. The coefficient of variation (CV) for lean body mass and body fat was < 2%.

Statistical method

Analyses were performed on runners with irregular menstrual function versus runners with regular menstrual function. Data were compared using Student's t-test. Values are given as Mean ± SEM.
Correlation analyses were performed according to Pearson. The significance level was set to α=0.05. SPSS 14.0 was used for the statistical analyses.

Results

Weight stability

During the previous year, one participant with regular menstrual function recorded a temporary weight gain of approximately 4 kg during the low-activity season. The weight returned to normal without any dietary intervention as the training activity escalated during the pre- competition period. All other participants were weight stable with weight fluctuations within ± 1 kg.

Nutrient intake

All participants reported intakes of meat and fish products during the observation period. Energy intakes in both groups of athletes (12.2 MJ in R and 9.9 MJ in IR) (Table 4a) were above estimated energy requirements of normally active/sedentary young women with similar height and weight.

Table 4a. Daily intake of macro nutrients (Mean ± SEM)

Group	N	Energy (MJ)	Fat (g)	Fat ($g \cdot kg^{-1}$)	Protein (g)	Protein ($g \cdot kg^{-1}$)	CHO (g)	CHO ($g \cdot kg^{-1}$)
R	10	12.2±0.7*	98±12*	1.7±0.2*	97±5	1.7±0.1	399±27	7.0±0.4
IR	10	9.9±0.4	61± 6	1.1±0.1	89±6	1.5±0.1	341±24	5.8±0.5

CHO = Carbohydrates

Table 4b. Daily intake of fat (Mean ± SEM)

Group	N	Saturated fat (g)	Monounsaturated fat (g)	Polyunsaturated fat (g)
R	10	35.2±2.7§	30.6±3.2 *	15.7±3.5
IR	10	25.2±2.9	19.0±2.2	9.1±1.4

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
* p=0.01 R vs. IR
§ p=0.02 R vs. IR

For some food items the detailed content of saturated/unsaturated fats was not provided by the manufacturers. These food items have been excluded from the calculations.

According to estimated energy requirements for female athletes, an amount of 2300-2500 kcal/day (9.6-10.5MJ/day) seems to be adequate to maintain weight balance, while endurance athletes may require more (up to 16.7 MJ per day) depending on training intensity and duration (Manore, 2002). The energy intake in participants with irregular menstrual function was in the lower range of the estimated requirements and significantly lower ($p = 0.01$) than in participants with regular menstrual function.

While intakes of protein and carbohydrates were similar and within normal range in the two groups, the intake of fat was significantly lower ($p = 0.01$) in the participants with irregular menstrual function compared with the participants with regular menstrual function. Estimated distribution between saturated and unsaturated fat is given in Table 4b.

Calculated intakes of minerals, water soluble vitamins and vitamin A were similar in the two groups, and above recommended levels (Table 5). Mean intake of Vitamin D was considerably lower in the participants with irregular menstrual function than in the group with regular menstrual function, but because of a large variability, statistical significance was not attained. Estimated dietary intake of vitamin E was significantly lower in the group with irregular menstrual function than in the group with regular menstrual function, and both groups had calculated

mean intakes lower than the recommended amounts of 15 mg per day (Manore, 2002)(Table 5).

Table 5. Dietary content of vitamins and minerals (Mean ± SEM)

Group	N	Iron (Mg)	Vitamin C (Mg)	Vitamin D (µg)	Vitamin A (µg)	Vitamin E (Mg)	Selenium (µg)	Thiamin (Mg)	Riboflavin (Mg)
EAR†		8.1	60	5-10	500	12	45	0.9	0.9
R	10	13.9±0.7	122±16	9±4	1828±372	9.2±1.4*	56±5	1.6±0.1	1.6±0.1
IR	10	13.0±1.1	120± 24	3±1	1361±321	5.0±0.7	47±6	1.5±0.1	1.6±0.1

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
* p<0.01 R vs. IR
† EAR: Estimated Average Requirements are given in bold figures, individual recommended daily intakes are given in normal figures. Values are according to the DRI reports from IOM, National Academy of Sciences 2002, for women between 19-50yrs. Recommended intakes of vitamin D in irregularly menstruating athletes are set according to requirements for estrogen deficient women (10µg)

Supplements

Two of the participants with regular menstrual function took cod liver oil, which is rich in unsaturated fatty acids and the vitamins D and E. The cod-liver oil was included in the present analysis of nutritional intake.
Extra iron supplementation was registered in three participants (Two R, One IR) and in addition, three participants (Two R, Two IR) took multivitamin pills (Vitaplex) containing 10mg Vitamin E per day. Additionally, one participant took Vitamin E supplements (100 mg per week). Due to an ongoing debate about the bioavailability of supplements, these were not included in the present analysis of nutritional intake.

Vitamin E status

There were significant differences in serum concentrations of α-tocopherol, regardless of whether the α-tocopherol was expressed as $\mu mol \cdot L^{-1}$ or as vitamin E index, which is the ratio between serum α-tocopherol and the sum of cholesterol and triacylglycerol ($p = 0.01$ and $p = 0.009$ respectively).
The mean α-tocopherol level in the group with irregular menstrual function was 15.7 ± 0.8 $\mu mol \cdot L^{-1}$, and in the group with regular menstrual function 27 ± 4 $\mu mol \cdot L^{-1}$. The corresponding ratios between serum α-tocopherol and the sum of cholesterol and triacylglycerol were IR:2.7 ± 0.3 $\mu mol \cdot (Mmol)^{-1}$ and R: 4.9 ± 0.7 $\mu mol \cdot (Mmol)^{-1}$. The levels of serum cholesterol (R: 4.7 ± 0.1, IR: 5.1 ± 0.3 $mmol \cdot L^{-1}$) and triacylglycerol (R: 0.94 ± 0.09, IR: 1.11 ± 0.14 $mmol \cdot L^{-1}$) were not significantly different and within normal range (Table 6a). Vitamin E status in the 13 participants participating in the haemolysis test did not significantly deviate from the total group. (Table 6b).

Table 6a. Serum levels of α-tocopherol, triacylglycerol and cholesterol (Mean ± SEM)

Group	N	α-tocopherol ($\mu mol \cdot L^{-1}$)	Triacylglycerol ($Mmol \cdot L^{-1}$)	Cholesterol ($\mu mol) \cdot (Mmol)^{-1}$	Vitamin E ratio (†)
R	10	27.3±3.6*	0.9±0.1	4.7±0.1	4.9±0.7*
IR	10	15.7±0.8	1.1± 0.1	5.1±0.3	2.7±0.2

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
* p<0.01 R vs. IR
† Vitamin E ratio= [α-tocopherol] / ([Triacylglycerol] + [Cholesterol])

Table 6b. Serum levels of α-tocopherol, triacylglycerol and cholesterol (Mean ± SEM) in participants participating in the haemolysis test

Group	N	α-tocopherol ($\mu mol \cdot L^{-1}$)	Triacylglycerol ($Mmol \cdot L^{-1}$)	Cholesterol ($\mu mol) \cdot (Mmol)^{-1}$	Vitamin E ratio (†)
R	6	31.3± 5.2	0.9±0.1	4.6±0.2	5.7±0.9
IR	7	15.7±1.13	1.2±0.2	5.2± 0.4	2.5±0.3

R = athletes with regular menstrual function.
IR = athletes with irregular menstrual function
* p<0.01 R vs. IR
† Vitamin E ratio= [α-tocopherol] / ([Triacylglycerol] + [Cholesterol])

Serum α-tocopherol levels tended to increase with increasing estimated intakes of vitamin E, but a significant correlation was not found in the present material (r= 0.413, p = 0.07). When supplements were included in the estimated dietary intake, the correlation did not improve (r=0,37, p=0,11).
Pre-exercise osmotic fragility of erythrocytes was not significantly related to resting serum α-tocopherol levels (r = - 0.37, p = 0.22, n = 13). Post-exercise osmotic fragility, however, increased significantly with decreasing serum α-tocopherol levels (r = - 0.58, p = 0.036, n = 13).

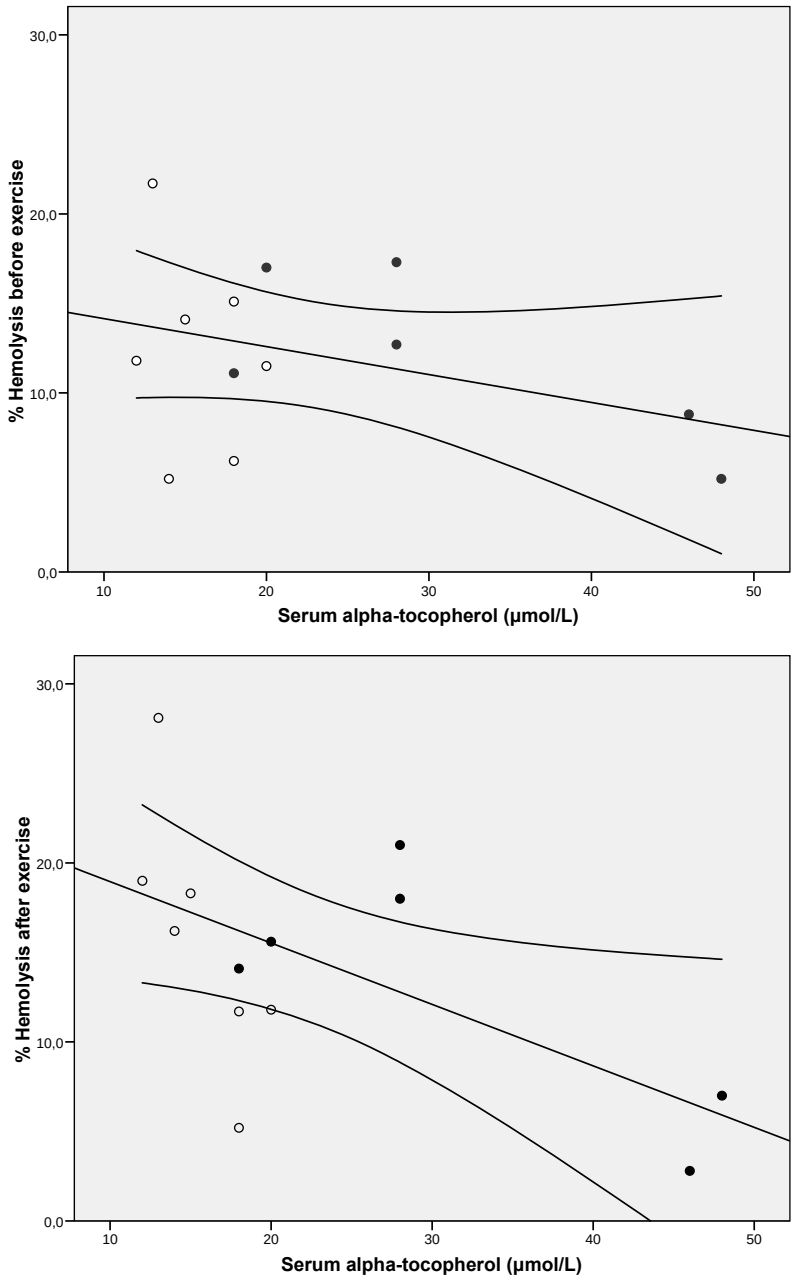

Figure 1a and 1b.
Percent haemolysis in 0,45% NaCl solution as a function of serum alpha-tocopherol (µmol/L) before and after exercise.
Figures show regression lines with 95% CI of the mean.
Open circles indicate participants with irregular menstrual function (IR) and filled circles indicate participants with regular menstrual function (R).
Three of the four participants taking Vitamin E supplements had less than 10% haemolysis after exercise, while the forth participant (IR) taking vitamin E supplements had inconspicuous results (serum alpha-tocopherol = 14 µmol/L and haemolysis 16,2%).

Discussion

There has been conflicting results regarding the benefits of Vitamin E supplements among athletes. Supplements do not seem to improve performance in people consuming adequate diets, and on the basis of nutritional investigations it has been assumed that the Vitamin E deficiency is not frequently encountered in the general population (Lukaski, 2004), although low intakes of vitamin E have been observed both in female and male athletes (Guilland et al., 1989). The weak association between estimated dietary intakes of vitamin E and serum levels of α-tocopherol is in accordance with previous reports (Ford et al., 1999; Guilland et al., 1989).

The participants with regular menstrual function seem to have normal serum levels of α-tocopherol, and data correspond well with the results in a study of female athletes and untrained females published in 1989 (Rokitzki, Berg, & Keul, 1989). In contrast, α-tocopherol levels in the participants with irregular menstrual function (15.7 ± 0.8 μmol/L), fell below the 10-percentile of a Swedish reference population (Ohrvall, Tengblad, & Vessby, 1993).

The low serum values in participants with irregular menstrual function in the present study could be a result of several factors. In particular, a low dietary content of Vitamin E, and a reduced bioavailability of the vitamin due to low dietary fat content should be considered, as the amount of alpha-tocopherol absorbed seems to vary in proportion to the amount of fat eaten with the vitamin (Bruno, Leonard, Park, Zhao, & Traber, 2006; Wolf, 2007). There is also reason to believe that endurance athletes may require more alpha- tocopherol than the general population, due to enhanced oxygen free radical formation during exercise (Jenkins, 1993). Serum levels of alpha- tocopherol as high as 36.8 $\mu mol/L^{-1}$ has been proposed in order to meet the oxidative stress of strenuous exercise (Takanami, Iwane, Kawai, & Shimomitsu, 2000).

Insufficiency or imbalance of essential food factors, combined with strenuous and enduring physical work, has been considered a basic cause for "amenorrhea as associated with war" (Benson, 1950). This was a frequent condition in Central Europe both during the First- and Second World War. Whether menstrual failure during war time was due to emotional stress, caused by a nutritionally inadequate diet, strenuous physical work, or a combination of several factors, is not known, but psychotherapy combined with supplies of wheat germ oil (Vitamin E) seemed to correct the dysfunction in a majority of the treated subjects (Whitacre & Barrera, 1944).

In contrast to extensive research on the significance of dietary carbohydrates and proteins for performance and health, the possible existence of a minimum requirement of fat in athletic nutrition has received little attention (Manore, 2002). A low fat content constitutes food with a low caloric density, and in view of the relatively high energy requirements in endurance athletes, the bulk of food needed for a sufficient energy intake might be difficult to consume. This may consequently lead to a negative energy balance, especially during intensive training periods (Tomten, 2006). A negative energy balance is considered a major risk factor for

menstrual disturbances (Loucks, 2003).
Secondly, as observed in the present study, the intake of fat-soluble vitamins may not reach the recommended level in a low fat diet and that in it self may be a health hazard.
Low serum values of α-tocopherol as observed in athletes with irregular menstrual function in the present study, as well as possible energy drain observed in previous studies might indicate that the aspect of a minimum requirement for dietary fat should be studied in more detail, also because post exercise osmotic fragility was inversely related to serum α-tocopherol levels.

Conclusions

Menstrual irregularities seem to be associated with a low fat intake and low levels of circulating α-tocopherol in female athletes. Furthermore, low α-tocopherol levels seem to render the red blood cells more susceptible to haemolysis in connection with physical activity.

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