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Effects of Alpha Lipoic Acid, Ascorbic Acid-6-Palmitate, and Fish Oil on the Glutathione, Malonaldehyde, and Fatty Acids Levels in Erythrocytes of Streptozotocin Induced Diabetic Male Rats

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Abstract In this research, it has been aimed to evaluate the improvement effects of alpha lipoic acid (ALA), ascorbic acid-6-palmitate (AA6P), fish oil (FO), and their combination (COM) on some biochemical properties in erythrocytes of streptozotocin (STZ)-induced diabetic male rats. According to experimental results, glutathione (GSH) level in erythrocytes decreased in diabetes ($P < 0.01$), D + ALA, and D + AA6P groups ($P < 0.001$). Malonaldehyde (MA) level increased in diabetes ($P < 0.05$), D + FO, and D + COM groups ($P < 0.001$), but its level in D + AA6P and D + ALA groups was lower in diabetes group ($P < 0.01$). Total lipid level in diabetes and diabetes plus antioxidant administered groups were higher than control. Total cholesterol level was high in diabetes and D + ALA groups ($P < 0.05$), but its level reduced in D + FO compared to control and diabetes groups, $P < 0.05$, < 0.001, respectively. Total triglyceride (TTG) level was high in the D + ALA ($P < 0.05$) and D + COM ($P < 0.001$) groups. In contrast, TTG level in blood of diabetes group was higher than diabetes plus antioxidant and FO administered groups ($P < 0.001$). According to gas chromatography analysis results, while the palmitic acid raised in diabetes group ($P < 0.05$), stearic acid in D + FO, D + ALA, and diabetes groups was lower than control ($P < 0.05$), oleic acid reduced in D + COM and D + FO groups, but its level raised in D + AA6P and D + ALA groups ($P < 0.01$). As the linoleic acid (LA) elevated in ALA + D, D + AA6P, and diabetes groups, linolenic acid level in diabetes, D + AA6P, and D + FO groups was lower than control ($P < 0.001$). Arachidonic acid (AA) decreased in D + ALA, D + AA6P, and diabetes groups ($P < 0.01$), but its level in D + COM and D + FO was higher than control ($P < 0.05$). Docosahexaenoic acid (DHA) increased in D + AA6P and D + COM ($P < 0.05$). While the total saturated fatty acid level raised in diabetes group, its level reduced in D + ALA and D + FO groups ($P < 0.05$). In contrast, total unsaturated fatty acid level in D + ALA and D + FO groups was higher than control ($P < 0.05$). In conclusion, present data have confirmed that the combination of the ALA, AA6P, and FO have improvement effects on the recycling of GSSG to reduced GSH in erythrocytes of diabetic rats, and in addition to this, oxidative stress was suppressed by ALA and AA6P, and unsaturated fatty acid degree was raised by the effects of ALA and FO. J. Cell. Biochem. 86: 530–539, 2002.

Key words: alpha lipoic acid; ascorbic acid-6-palmitate; fish oil; erythrocytes; lipid composition; reduced glutathione; malonaldehyde; fatty acids; diabetic male rats

Diabetes is a complex syndrome involving severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis and lipid metabolism [Horrobin, 1993, Wolf, 1993, Severcan et al., 2000]. The disease is generally broken down into two major groups as insulin dependent and non-insulin dependent diabetes [Taylor and Agius, 1988; Wolf, 1993]. In the former type, also referred as type I diabetes, there is total or near total loss of insulin secretion from the islets of Langerhans in the pancreas resulting in disinhibition of gluconeogenesis, soaring blood glucose levels, and gross loss of muscle and fat [Wolf, 1993]. Since Δ-6

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desaturation pathway is inadequate in diabetes, essential fatty acid metabolism is acquired [Horrobin, 1993]. It has been reported that diabetic animals required much more linoleic acid (LA) than normal animals. The reason of this is greatly impaired Δ-6 desaturase enzyme activity in diabetic animals. Because linoleic acid (LA, 18:2) exerts most of its biological effects by being converted into gamma linoleic acid (GLA). Because of impairing Δ-6 desaturation in diabetes, LA concentration is almost always normal or slightly above normal, whereas the concentrations of LA metabolites such as eicosatrienoic (20:3) and arachidonic (AA, 20:4) acids are consistently below normal [Horrobin, 1993]. When diabetics are treated with continuous subcutaneous insulin therapy, the concentrations of LA major metabolites rise significantly in cells. The main problem in the management of diabetes is the development of long-term damage to the retina, the kidneys, the cardiovascular system, and the peripheral nerves [Horrobin, 1993].

In this research, the effects of DL-alpha lipoic acid (ALA), ascorbic acid-6-palmitate (AA6P), fish oil (FO), and their combination (COM) on the erythrocytes were examined. ALA is a vital cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids. It plays an important role in lipid biosynthesis by replacing coenzyme. In addition to its cofactor role, ALA is a powerful antioxidant and possesses numerous cellular functions as well as beneficial effects in conditions with elevated oxidative stress [Arivazhagan et al., 2000]. Vadoevich [1983] found that ALA treatment raised the content of LA (18:2) and AA (20:4) acids and to decrease the content of myristic acid in blood of patients with coronary heart disease and essential hypertension. Jayanthi and Varalakshmi [1992] reported that ALA treatment reduced tissue cholesterol and triglyceride levels significantly and raised phospholipids in calculogenic rats. AA6P is a lipophilic derivative of ascorbic acid. It is a good antioxidant in model systems and is also effective in cellular systems. It has been suggested that the antioxidant effect of AA6P is much better than ascorbic acid and α-tocopherol, both in vivo and in vitro systems [Nostro, 1997]. Ross et al. [1999] have stated that erythrocyte-bound AA6P protect membrane α-tocopherol from oxidation by both ferricyanide and a water-soluble free radical initiator, suggesting that the derivative either reacted directly with the exogenously added oxidant, or that it was able to recycle the α-tocopherol radical to α-tocopherol in the cell membrane. AA6P also partially protect *cis*-prinaric acid from oxidation when this fluorescent fatty acid was intercalated into the membrane of intact cells. FO is a rich source of long-chain n-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA: 20:5) [İbrahim et al., 1999]. The PUFAs have gained importance in prostaglandin metabolism and also they have essential role in membrane function [Kamada et al., 1986]. It has been reported that n-3 fatty acids have exerted a variety of beneficial effects by inhibiting AA (20:4) by providing an alternative substrate for lipid mediator synthesis, i.e., EPA (20:5) [Hammes et al., 1996]. However, more intake of FO is associated with increased susceptibility of membranes to oxidation and increased requirement for antioxidants [Ibrahim et al., 1999]. The tripeptide GSH is the thiol compound, which present the highest concentration in cells of all organs. GSH has many physiological functions including its involvement in the defense against reactive oxygen species [Dringen, 2000]. It is major cellular thiol, participating in cellular redox reactions and together formation [Sies, 1999]. GSH depletion to about 20–30% of the total glutathione (GSH) levels can impair the cell defense against the toxic actions of reactive oxygen species and may lead to cell injury and death [Sen, 1997]. Because of powerful antioxidant and metabolic effects of ALA and AA6P and biological effects of PUFAs in FO content, it has been purposed to evaluate their therapeutic effects on lipid and fatty acid composition, GSH, and malonaldehyde (MA) levels in the erythrocytes of streptozotocin (STZ) induced diabetic male rats.

MATERIALS AND METHODS

Animals

Forty-five adult male Wistar rats were used in this study. At the start of the experiment, the weights of the rats were between 250.65 ± 45.12 and 350.34 ± 67.45 g and their ages were 5 weeks. The rats were randomly divided into six groups and kept at room temperature, 20°C. These animals were fed ad libitum a diet including ingredients shown in Table I, during the experiment. The first group was used as a

TABLE I. Diet Composition for Experimental Rats

Ingredients	%
Wheat	10
Corn	22
Barley	15
Wheat bran	8
Soybean	26
Fish flour	8
Meat-bone flour	5
Pelted	5
Salt	0.8
Vitamin and mineral mixture	0.2

Vitamin A, D_3, E, K, B_1, B_2, B_6, B_{12}, nicotinamide, folic acid, biotin, choline chloride, Mn, Fe, Zn, Cu, I, Co, Se, antioxidant and Ca.

control (n = 8), the second group diabetes (D) (n = 10), the third group diabetes + alpha lipoic acid (D + ALA) (n = 8), the fourth group diabetes + ascorbic acid-6-palmitate (D + AA6P) (n = 5), the fifth group diabetes + FO (D + FO) (n = 6), and the sixth group diabetes + the combination of ALA, AA6P, and FO (D + COM) (n = 8). Rats in D, D + ALA, D + AA6P, D + FO, and D + COM groups were made diabetic using a single intraperitoneal (i.p.) injection of 45-mg/kg STZ in citrate buffer (pH = 4.5). Control group rats were injected intraperitoneally with buffer alone. Two days later, after administration of STZ, tail vein blood glucose was measured in all animals. The level of blood glucose, which was 250 mg/dl and above was considered diabetics. Rats in D, D + ALA, D + AA6P, D + FO, and D + COM groups were injected subcutaneously daily between 8–12 IU/kg NPH (Neutral Protamine Hegadorn) insulin (Insulatard HM 100 IU/ml Nova Nordisk). In addition, the rats in D + ALA group were injected intraperitoneally 12 mg/kg ALA four times per week, in D + AA6P group they were injected intraperitoneally 48 mg/kg AA6P, in D + FO group, injected intraperitoneally 1 ml/kg FO, and in D + COM group they were injected 25 mg/kg ALA + 25 mg/kg AA6P + 0.80 ml/kg FO.

This administration was done for a period of 8 weeks. The latest dose was administered 12 h before the operation. At the end of this period, after overnight fasting; each experimental rat was anesthetized with ether. Then, blood samples were taken from heart vein into ETDA vacutainer tubes via injector, and were separated from sera by centrifugation process at 4°C.

Preparation of Erythrocyte Pellets

Blood from rats was taken into ETDA vacutainer tubes. Erythrocyte pellets were separated from plasma and the leukocyte layer by centrifugation in 4,000 rpm. Then, erythrocyte pellets were washed three times with cold (4°C) phosphate-buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH = 7.4). These pellets were used immediately after isolation for biochemical analyses.

Determination of GSH in Erythrocytes

Reduced GSH in erythrocytes was assayed by acting with 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) as described [Akerboom and Sies, 1981; Lee and Churg, 1999]. Briefly erythrocyte pellets were homogenized in cold 1-ml 20 mM ETDA and 2-ml PBS solution. The mixtures were centrifuged in 4,000 rpm and were taken 200 μl supernatant. After deproteinization with 1.67% metaphosphoric acid (Sigma, St. Louis, MO), an aliquot of supernatant was allowed to react with 1 ml 0.04% DTNB (Sigma) solution. The yellow product was detected and quantified spectrophotometrically at 416 nm. Pure GSH was used as standard for establishing the calibration curve.

Determination of Lipid Peroxidation Products (LPO)

The level of LPO in erythrocytes was assayed by measuring the reaction products between MA and thiobarbituric acid (TBA) as described [Ohkawa et al., 1979]. Briefly, erythrocyte pellets were homogenized in cold PBS and the mixture was centrifuged in 4,000 rpm and were taken 200 μl supernatant. LPO in the crude extract was precipitated and washed with 10%-phosphothungustic acid. After resuspending in distilled water, LPO was allowed to react with 0.67% TBA at 95°C for 1 h. The reaction mixture was cooled to room temperature and reaction product was extracted with equal volume of n-butanol. The product was measured at the 535 nm in a spectrophotometer. 1,1,3,3-Tetraethoxypropane (malonaldehyde bis[diethyl acetal]) (Merck, Darmstadt, Germany) was used as standard for setting up the calibration curve.

Lipid Extraction

The total lipid was extracted with chloroform–methanol (2:1, v/v) by the method of Folch et al. [1957] as previously described [Christie, 1990]. Erythrocyte pellets were homogenized with the mixture of chloroform–methanol (2:1, v/v) in MICRA D 8 homogenizator. Non-lipid contaminants

were removed by washing with 0.88% KCl solution. The extracts were evaporated in a rotary evaporator flask and dissolved in n-hexane and stored at −25°C until further analysis.

Determination the Levels of Total Lipid, Cholesterol, and Triglyceride

Total lipid was determined according to the method of Frings et al. [1972] as previously described [Yilmaz et al., 1997a; Yilmaz et al., 1997b]. Each lipid extract of 10 μl was taken and treated with 500 μl of concentrated sulphuric acid and left at boiling water for 10 min. Then, 5 ml of phosphovanilin reagent was added and the mixture was incubated at 25°C for 20 min. After incubation, the optical densities of samples were read at 540 nm against blank. In addition, standard solution was prepared in the same condition from USA grade olive oil (Sigma, St. Louis, MO). The total lipid level was calculated according to the standard curve, prepared from standard.

Total cholesterol level was determined with the Liebermann Burchard reaction as described by Christie [1982]. The reagent was prepared by mixing with acetic anhydride (60 ml) and glacial acetic acid (40 ml). A sample of 200 μl was treated with 5 ml of reagent and 0.5 ml of concentrated sulphuric acid was added. After that, these mixtures were incubated at 25°C for 20 min and read at 560 nm. The calculation was made according to the standard curve, which was prepared from pure cholesterol standard (Merck).

Total triglyceride (TTG) was carried out by the periodate oxidation method as described [Gottfried and Rosemberg, 1973; El-Sayed et al., 1984]. Each sample of 250 μl was taken and evaporated to dryness under nitrogen and the residue was saponified by heating at 70°C for 15 min in stoppered tubes with 1 ml of 0.63% metanolic KOH. After cooling, the solution of 1 ml 0.2 N sulphuric acid was added, and followed by 0.2 ml 0.6% $NaIO_4$ in 5% acetic acid solution. The solution was mixed and allowed to stand for 10 min. Then, the mixture was added to solution 5 ml of chromotropic acid reagent (0.2% w/v sodium salt in 60% v/v sulphuric acid). After stoppering, the tubes were heated at 100°C for 15 min and then cooled. The optical density of each sample was read at 570 nm. A calibration curve was obtained by taking glycerol oleate (Sigma).

Fatty Acid Analysis

Fatty acids in lipid extracts were converted to methyl esters by using 2% sulphuric acid (v/v) in methanol [Christie, 1990, Yilmaz et al., 1997a; Yilmaz et al., 1997b; Yilmaz et al., 1997c]. Fatty acid methyl ester forms were extracted with n-hexane. These methyl esters were then separated and quantified by using a gas chromatography equipped with a flame-ionization detector (FID) (600 series gas chromatography) attached to a Unicam 4815 computing recorder. Chromatography was performed with a BPX capillary column (25 m in length and 0.22 mm in diameter) and using hydrogen as carrier gas (flow rate 2.5 ml/min). The temperatures of the column, detector, and injection port were 190, 200, and 220°C, respectively. A Unicam recorder automatically computed retention times and peak areas and length. Identification of the individual methyl esters was performed by frequent comparison with authentic external standard mixtures analyzed under the same conditions. In order to calculate the fatty acid proportions, heptadecanoic acid (margaric acid) was used as internal standard.

Statistical Analysis

Experimental results were reported as mean ± SD. Statistical analysis was performed using SPSS Software. The variance analysis (ANOVA) and LSD test were used for comparison between the control and diabetes plus antioxidant administered groups.

RESULTS

Levels of MA and GSH in Erythrocyte

While the GSH level in erythrocytes did not differ between control and D + COM groups, its level decreased in diabetes ($P < 0.05$), D + ALA ($P < 0.01$), and D + AA6P groups ($P < 0.001$) (Table II). The level of MA in erythrocytes increased slightly in diabetes group ($P < 0.05$), but its level in D + FO and D + COM groups was higher than control ($P < 0.001$). In contrast, the amount of MA reduced in D + AA6P and D + ALA groups ($P < 0.01$) (Table II).

Levels of Total Lipid, Cholesterol, and Triglyceride in Erythrocytes

The level of total lipid in erythrocytes or diabetes and diabetes antioxidants administered groups was higher than control groups.

TABLE II. Level of GSH, MDA in Erythrocytes and TTG in Blood of Tail Vein

Groups	GSH (μg/g wet cell pellet)	MDA (μg/g wet cell pellet)	TTG in Blood (mg/dl)
Control	19.49 ± 1.37*	0.547 ± 0.105	65.20 ± 3.35*
Diabetes	14.44 ± 1.03**	0.696 ± 0.206**	146.00 ± 10.40****
D + ALA	10.97 ± 0.98***	0.300 ± 0.063***	94.78 ± 8.19**
D + AA6P	6.84 ± 0.40****	0.310 ± 0.068***	113.50 ± 2.38**
D + FO	7.85 ± 0.95****	0.940 ± 0.116****	116.00 ± 14.02**
D + COM	20.06 ± 2.20*	1.143 ± 0.269****	74.50 ± 10.23*

*$P > 0.05$.
**$P < 0.05$.
***$P < 0.01$.
****$P < 0.001$.

According to these results, the level of lipid increased in diabetes, D + FO and D + ALA ($P < 0.01$), D + AA6P and D + COM groups ($P < 0.001$). Total cholesterol level was partly high in diabetes and D + ALA groups ($P < 0.05$), but no differences were found between D + AA6P and control groups. The cholesterol level reduced in D + FO as compared to control and diabetes groups, $P < 0.05$, < 0.001, respectively. TTG level was not found to differ among the diabetes, D + FO, and D + AA6P and control groups, but its level raised in D + ALA ($P < 0.05$) and D + COM ($P < 0.001$) groups (Table III).

At the end of administration time, triglyceride level in tail vein blood was measured by Accutrent GCT via glucose oxidase. According to this result, the triglyceride level in blood was higher in diabetes group than other diabetes plus antioxidant administered and control groups ($P < 0.001$). There were no found differences between D + COM and control groups, but its level reduced significantly in diabetes plus antioxidant administered groups in comparison to diabetes group (Table II).

Proportions of Fatty Acids in Erythrocyte Membrane Lipid Composition

According to gas chromatography analysis results, it was found in erythrocytes fatty acid composition that myristic (14:0), pentadecanoic (15:0), palmitic (16:0), stearic (18:0), oleic (18:1), LA (18:2), LNA (18:3), AA (20:4), and DHA (22:6) acids. According to these results, myristic acid increased in D + ALA and D + AA6P groups, $P < 0.01$, < 0.001, respectively, but no difference was found in the groups of diabetes and control. In contrast, it was observed that the level of myristic acid decreased in D + FO group ($P < 0.01$). Pentadecanoic acid level in diabetes and D + COM and D + FO groups was higher than control group ($P < 0.01$, < 0.001). While the palmitic acid rose in diabetes group as compared to control group ($P < 0.01$), no differences were found between control and diabetes plus antioxidant administered groups. Stearic acid reduced in D + FO, D + AA6P, and diabetes groups ($P < 0.05$), but there was no difference among the control and D + ALA and D + COM groups (Table IV). Oleic acid level in D + COM and D + FO groups was lower than control ($P < 0.01$), but no differences was found between control and diabetes groups ($P < 0.01$). In contrast, this fatty acid increased D + AA6P and D + ALA groups ($P < 0.01$). LA (18:2) level found no differences among the control, D + FO, and D + COM groups, but its level rose in D + ALA and D + AA6P groups. LNA (18:3) level in diabetes and diabetes plus antioxidant administered groups was lower than control group. But it was observed that LNA level in D + ALA and D + COM groups is close to control group. AA (20:4) decreased in D + ALA and diabetes groups compared to control ($P < 0.01$), but its level was high in D + COM group ($P < 0.05$). There was no difference between control and D + FO. While the DHA (22:6) increased in D + AA6P and D + COM groups, $P < 0.001$, < 0.05, respectively, no differences was found between control and diabetes plus antioxidant administered groups (Table IV). As the proportion of total saturated fatty acid (ΣSFA) increased in diabetes group, its level decreased in D + ALA and D + FO groups ($P < 0.05$) (Table IV). However, the level of total unsaturated fatty acid (ΣUSFA) was higher in D + ALA and D + FO groups ($P < 0.05$), but its level in diabetes group was lower than control group ($P < 0.05$) (Table V).

DISCUSSION

The erythrocyte was an early model for studies of oxidative stress [Stern, 1985]. It should be prone to oxidative reactions, because

TABLE III. Levels of TL, TCHOL, and TTG in Erythrocytes (mg/g Wet Cell Pellet)

Groups	TL	TCHOL	TTG
Control	67.74 ± 6.45	16.51 ± 1.52*	22.36 ± 0.84*
Diabetes	119.39 ± 23.53**	19.71 ± 1.43**	22.08 ± 0.80*
D + ALA	150.37 ± 27.25***	19.27 ± 1.56**	25.66 ± 0.84**
D + AA6P	267.97 ± 32.37****	16.49 ± 1.51*	22.27 ± 0.05*
D + FO	143.90 ± 23.25***	15.59 ± 0.43*	22.19 ± 0.28*
D + COM	258.64 ± 35.20****	16.89 ± 2.11*	33.52 ± 0.30****

*$P > 0.05$.
**$P < 0.05$.
***$P < 0.01$.
****$P < 0.001$.
TL, total lipid; TCHOL, total cholesterol; TTG, total triglyceride.

of relatively high oxygen tensions, presence of hemoglobin, and a plasma membrane rich in PUFAs. Erythrocytes have many oxidant defense systems. Many genetic and acquired conditions subject erythrocytes to oxidant damage [Van Asbectk et al., 1985]. Present data showed that the amount of MA in erythrocytes was high in diabetes, D + FO, and D + COM groups, but its level was low in D + ALA and D + AA6P groups (Table II). It has been suggested that ALA improves nerve blood flow, reduces oxidative stress, and improves distal nerve conduction in experimental diabetic neuropathy. Also, it has been demonstrated that ALA will reduce oxidative stress in diabetic peripheral nerves and improve neuropathy [Hendrickson et al., 1995]. Nagamatsu et al. [1995] have determined that ALA did not affect the nerve blood flow of normal nerves, but improve that of diabetic neuropathy in a dose-depended manner. AA6P is a lipophilic antioxidant and it is bound to erythrocyte membranes where it plays oxidation–reduction reaction [Ross et al., 1999]. It has been stated that AA6P has more powerful antioxidant effect than L-ascorbic acid and α-tocopherol, in vitro and in vivo systems [Nostro, 1997]. FO is the most important source of n-3 fatty acids. Though n-3 fatty acids have many therapeutic biological effects, but they are susceptible to oxidation, and the resulting products are toxic to cell [Ibrahim et al., 1999]. Increased intake of FO is associated with elevated susceptibility of membranes to oxidation and an increased requirement for antioxidants [Ibrahim et al., 1999]. Since erythrocytes are, perhaps, the cells most exposed to peroxidation damage by free radical during their relatives short life in which no protein synthesis occur, these cells whose principal role is to carry oxygen to the tissues and organs, come in close contact with free radical from various sources [Constantinescu et al., 1998, Giron et al., 1999]. It was found that the level of MA in D + FO and D + COM groups was higher (Table II). This increase may be due to high polyunsaturated fatty acid content in FO. In addition, although the level of MA in diabetes group was high, its level in diabetes group was lower than D + COM group. This result shows that diabetic rats are well controlled during the experiment time. Because it has been injected insulin subcutaneously as in diabetes and diabetes plus antioxidant administered groups. Yagi [1987] reported that the lipid peroxide level in plasma of poorly controlled diabetics was higher than that of well-controlled ones.

TABLE IV. Proportions of Saturated Fatty Acids in Fatty Acid Composition of Erythrocytes (%)

Groups	14:0	15:0	16:0	18:0	ΣUSFA
Control	2.92 ± 0.72*	2.09 ± 0.44	58.08 ± 2.31*	3.27 ± 0.78*	66.23 ± 1.07*
Diabetes	2.89 ± 0.52*	2.70 ± 0.40***	61.10 ± 1.70**	2.38 ± 0.42**	69.88 ± 2.15**
D + ALA	3.70 ± 0.44***	1.97 ± 0.55**	56.33 ± 2.23*	3.00 ± 1.06*	64.87 ± 1.52**
D + AA6P	5.13 ± 0.39****	1.58 ± 0.23**	57.29 ± 1.51*	2.23 ± 0.21**	66.86 ± 1.57*
D + FO	1.53 ± 0.41****	2.72 ± 0.70***	58.22 ± 2.66*	2.53 ± 0.42**	63.60 ± 1.62**
D + COM	3.01 ± 0.48*	4.83 ± 0.13****	57.86 ± 4.22*	3.18 ± 0.23*	66.66 ± 2.38*

*$P > 0.05$.
**$P < 0.05$.
***$P < 0.01$.
****$P < 0.001$.

TABLE V. Proportions of Unsaturated Fatty Acids in Fatty Acid Composition of Erythrocytes (%)

Groups	18:1	18:2	18:3	20:4	22:6	ΣUSFA
Control	10.58 ± 0.74*	7.67 ± 1.38*	1.93 ± 0.19	11.13 ± 0.87*	2.47 ± 0.21*	33.77 ± 2.44*
Diabetes	10.85 ± 0.73*	9.42 ± 0.26**	1.08 ± 0.10****	7.25 ± 1.11***	2.51 ± 0.40*	30.12 ± 2.43**
D + ALA	13.46 ± 1.40***	9.21 ± 1.24**	1.41 ± 0.39***	8.39 ± 0.99***	2.75 ± 0.94*	35.13 ± 1.83**
D + AA6P	12.88 ± 1.55***	9.24 ± 0.81**	1.26 ± 0.58****	4.20 ± 0.52****	3.86 ± 0.41**	33.14 ± 1.82*
D + FO	7.90 ± 0.73***	8.79 ± 0.09*	0.80 ± 0.12****	13.48 ± 2.45**	2.77 ± 0.42*	36.40 ± 1.64**
D + COM	8.65 ± 1.94***	8.82 ± 0.85*	1.35 ± 0.33***	13.18 ± 1.48**	3.09 ± 0.25**	33.34 ± 2.21*

*$P > 0.05$.
**$P < 0.05$.
***$P < 0.01$.
****$P < 0.001$.

In contrast to increase of MA in D + COM group, the GSH level was found similar to control group. In addition, GSH level in D + COM group was higher than diabetes and diabetes plus antioxidant groups (Table II). In erythrocyte, GSH antioxidant system is very important and plays a fundamental role in cellular defense against reactive free radicals and other oxidant species [Giron et al., 1999]. GSH plays a key role in protecting cells against electrophiles and free radicals. This is due to the nucleophilicity of the SH group and to the high reaction rate of thiols with free radicals [Navarro et al., 1997]. Intracellular synthesis of GSH is a tightly regulated two-step process, both of which are ATP dependent. It is also generated intracellularly from its oxidized form glutathione disulfide (GSSG) by glutathione disulfude reductase activity in the presence of NADPH [Sen, 1997].

It was suggested that the lowering of GSH level in erythrocyte, the activity of glucose 6 phosphate dehydrogenase (G6PD) is decreased. This enzyme is important in the pentose phosphate pathway that generates NADPH. Reduced GSH maintains cell membrane sulfhydryl groups and other structural protein in stable form. NADPH required for GSH generation is supplied by G6PD [Arivazhagan et al., 2000]. When there is a reduction of GSH oxide to GSSG, cells are protected from the damage free radical and lipid peroxides [Exner et al., 2000]. The most sensitive and reliable indicator of oxidative stress was reduction in reduced GSH, which was significantly reduced in diabetes [Esteve et al., 1999]. In addition, GSH consists of functionally related to some enzymes. From the enzymes, Se-GPx protects cells from the lipid hydroperoxides damages, which is produced by phospholipase A2 from free fatty acid in membranes [Halliwell and Chirico, 1993]. Though erythrocyte defense system against cellular oxidants is complex and efficient, free radicals generated in cell membranes are relatively sequestered by the cell's antioxidant system [Fung and Zhang, 1990].

In lipid composition of erythrocytes, total lipid level was found to increase in D + AA6P and D + ALA and D + COM groups (Table III). Raised level of lipid in these groups may be due to the effects of AA6P and ALA. Jacob et al. [1999] have stated that ALA has favorable effects on glucose metabolism. When the ALA was treated to diabetic rats for 2 weeks, the effects of ALA led to the normalization of glucose uptake and glucose utilization and consequently to the normalization of oxygen uptake [Strodter et al., 1995]. In addition, it has been reported that ALA is able to increase glucose uptake in vitro and enhanced glucose uptake by cells serves as a fuel for both the pentose phosphate shunt and oxidative phosphorylation, thus bringing the cellular levels of NADPH and NADH [Arivazhagan et al., 2000]. It has been shown in different experiments and animal models that the effects of ALA both scavenge and enhance glucose transport [Jacob et al., 1999]. In addition, Qu et al. [1995] have suggested that ALA is a drug used for the treatment of diabetic polyneuropathy and acts as an antioxidant.

The lipid level in D + FO group was lower than D + ALA and D + AA6P groups (Table III). Decrease of the lipid level in this group may be due to antilipidemic effects of n-3 PUFAs in FO content. It has been suggested that n-3 PUFAs reduce the lipid biosynthesis in different tissues of body. Yosefy et al. [1999] have reported that n-3 PUFAs in FO content lead to a reduction in blood pressure and serum triglycerides, in addition to normalization of the hypercoagulate state in subjects with obesity hypertension and dyslipidemia without diabetes. While the

cholesterol level in D + ALA group was higher, its level in D + FO was lower than control group (Table III). Cholesterol and fatty acids are structural components for the membranes of all cells [Sinclair, 1984]. Although cholesterol is a structural component of cells, it has some disadvantages for the cell functions when its level elevates in the cells [Gurr and Harwood, 1990; Mathew and Van Holde, 1990; Kamada et al., 1986]. When the level of cholesterol rises in cells, its hydroxyl group lie to phase of water and lipid, and its hydrophobic section soluble in lipid phase. Consequently, the movement and fluidity of membrane cell is inhibited by high cholesterol level. As known, PUFAs in phospholipids of membranes play important role in membrane fluidity and movement [Sinclair, 1984; Gurr and Harwood, 1990; Kamada et al., 1986].

The cholesterol level in D + AA6P and D + COM groups was not found to differ in comparison with control group. According to results from present data, it can be said that AA6P is an important antioxidant for erythrocytes. Ross et al. [1999] have stated that AA6P play important role in erythrocyte defense system. Increase of cholesterol and triglyceride in D + ALA group may be due to the effects of ALA on the energy metabolism. ALA is used as cofactor in metabolic reactions and it plays important role in lipid biosynthesis [Hagen et al., 1999; Arivazhagan et al., 2000]. In addition, it has been stated that ALA has enhanced on the energy metabolism, which produces ATP from glucose [Packer et al., 1995; Kabir et al., 1998].

According to the results from gas chromatography analysis, the fatty acid composition of the erythrocytes consists of acids such as 14:0, 15:0, 16:0, 18:0, 18:1, LA (18:2), LNA (18:3), AA (20:4), and DHA (22:6) acids (Tables IV–V). While 14:0 increased in D + AA6P and D + COM groups, its level decreased in D + FO group. 16:0 rose in diabetes group, but 18:0 decreased in diabetes and D + FO groups. 16:0 is a key fatty acid for fatty acid synthesis. In this process, many different fatty acids are formed by elongation and desaturation of palmitate, by different desaturases activities. Fatty acid synthesis is a major metabolic pathway for the provision of energy reserves and cellular structural components. This pathway is regulated by complex nutrition and hormones [Kabir et al., 1998]. Increase of 16:0 in diabetes group may be due to the effects of insulin. In spite of injection of insulin to diabetes plus antioxidant administered groups, there were no differences between control and these groups. The reason for this may be due to the effects of antioxidants and FO. Antioxidants have the regulatory effects on the fatty acid synthesis, and FO has the antilipidemic effect. AA (20:4) was found high in D + FO and D + COM groups, and DHA (22:6) was high in D + AA6P and D + COM groups. Increase of AA (20:4) in D + FO and D + COM groups, and DHA (22:6) in D + AA6P and D + COM groups may be due to improvement effects of AA6P and ALA on the fatty acid desaturation system in cells.

In our results, it was found that the level of LA (18:2) was high in diabetes group, but AA (20:4) was low. Brenner et al. [2000] have stressed that the activities of fatty acid desaturases, such as delta 9, delta 6, and delta 5, were depressed in STZ induced diabetic rats. It has been stated that the concentration of LA in diabetes is almost slightly above, whereas the concentrations of its metabolites such as 20:3, AA (20.4) are consistently below normal [Horrobin, 1993]. Also, Shin et al. [1995] found that the levels of LA and 18:0 were high and 16:0 and AA (20:4) were low in diabetic rats. These authors have stressed that these alterations were persistently observed after 48 h of insulin treatment and the activities of delta 6 desaturase in diabetic rats were 68% of those of controls and increased to 119% of controls after insulin treatment. Tilwis et al. [1986] have suggested that the continuous subcutaneous insulin in diabetes increased significantly the concentrations of LA (18:2) metabolites such as 20:3, 20:4, and 20:5. Huang et al. [1987] and Nishida et al. [1998] have shown that the proportions of monounsaturated and PUFAs are increased in different tissues of the body by delta 4, delta 5, delta 6, and delta 9 desaturase enzymes.

In conclusion, present study has shown that the administration of ALA, AA6P, and FO to diabetic rats has effects of improvements on the recycling of oxide glutathione (GSSG) to reduce GSH in erythrocytes, and oxidative stress was suppressed by ALA and AA6P, and unsaturated fatty acid degree was raised by the effects of ALA and FO.

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