

Effect of nutrient antioxidants on oxidative stress indicators in patients with alopecia areata

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الخلاصة:

يعرف داء الحاصة البقعية بأنه تساقط الشعر على شكل بقع دائرية ملساء, السبب الحقيقي لهذا المرض غير معروف لحد الآن. اشارت بعض الدراسات الحديثة إلى دور الجذور الحرة في امراضية داء الحاصة البقعية ودور مانعات الأكسدة في حماية الجهاز المناعي للجسم من التأثير الضار لهذه الجذور الحرة. ان أي خلل في التوازن بين تكوين الجذور الحرة والمستوى الداخلي لمانعات الأكسدة الطبيعية للجسم يؤدي إلى حدوث الأجهاد التأكسدي مما يؤدي إلى أمراض كثيرة منها داء الحاصة البقعية.

صممت هذه الدراسة السريرية لدراسة دور مانعات الأكسدة في حماية الجسم من التأثير الضار للجذور الحرة, قياس مستوى العناصر النادرة (الزنك و النحاس) في مصل الدم في كل من المرضى و الأصحاء وتأثير فترة العلاج باستخدام مانعات الأكسدة (فيتامين أ, ه و ج) على كل من : مؤشرات الإجهاد التأكسدي , مستوى العناصر النادرة و سرعة نمو الشعر لمرضى داء الحاصة البقعية.

اظهرت نتائج الدراسة انخفاضاً واضحاً في المستوى الأساسي لمؤشرات الإجهاد التأكسدي و مستوى الزنك في مرضى داء الحاصة البقعية مقارنة بالأصحاء بينما مستوى النحاس أعلى بصورة ملحوظة منه في الأصحاء, كما إن العلاج باستخدام مانعات الأكسدة أدى إلى تحسن ملحوظ في المستوى الداخلي لمانعات الأكسدة الطبيعية الموجودة داخل الجسم , زيادة نسبة الزنك , تقليل نسبة النحاس وتصحيح نسبة عنصر الزنك إلى النحاس.

التأثير الأكثر أهمية من الناحية السريرية لمانعات الأكسدة هي الزيادة الملحوظة في سرعة نمو الشعر للمرضى المشمولين بهذه الدراسة (مرضى الحاصة البقعية) والذي من الممكن أن يعزى إلى اخماد حالة الإجهاد التأكسدي المصاحبة للمرض.

Abstract

People with alopecia areata lose their scalp hair in smooth round patches typically causing bald spots about an inch (2cm) across. Recently Al-Jaff et al demonstrate the association of free radicals in the pathogenesis of alopecia areata, and the role of nutrient antioxidants in protecting the immune system from the damaging effect of these radicals. Any imbalances between free radical production and antioxidant deficiency can result in an injury.

This clinical study designed to study the role of nutrient antioxidants in protecting the body from damaging effect of radicals and the effect of two month treatment with antioxidants (vitamin A, E, &C) on oxidative stress variable levels and their effect on rate of hair growth in patients with alopecia areata. Treatment with antioxidants significantly elevated body antioxidant defenses component levels, increased Zn levels and decreased

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Cu levels and corrected the imbalance in these trace metals metabolism in alopecia after 1 and 2 months of treatment compared to pretreatment values. The most important point is the clinical significance of antioxidants in improving the hair response of patients with alopecia areata; this may be due to an inhibition of oxidative stress associated with the state of the disease.

Introduction

Alopecia areata (AA) simply is sudden patchy hair loss. Free radicals are any molecules that contain one or more unpaired electrons and often extremely reactive, therefore short lived. The exact cause of alopecia areata is unknown; however researchers believed that it is an auto-immune condition (1, 2, 3, and 4).

An antioxidant can be defined as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits the oxidation of that substrate"(5). The physiological role of antioxidants is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals.

In a clinical study, Al-jaff et.al (2001) demonstrated for the first time, the association of lipid peroxidation in pathogenesis of alopecia and the role of some antioxidants like (vitamin A, E& C) in protecting the immune system from the oxidative damage produced by the disease(6).it is well known that there are free radicals formed naturally in the body which may have negative effects when they damage proteins, lipids and nucleic acids(6).

The present study was conducted to investigate the effects of the administration of nutrient antioxidants combination treatment on oxidative stress indicators level in patients with alopecia areata by trying antioxidants alone as the main therapy rather than to recommend using it with traditional treatment as an adjuvant therapy. This could offer interesting information's regarding the possible role of reactive oxygen radicals in the pathogenesis of alopecia areata.

Subjects and methods

1- Subjects

A-Study group: comprised of total of 44 subjects, 25 normal controls age (10-40) years old and 19 cases with AA. Patients involved in this study were under dermatologist supervision. They were non-smokers, non-alcoholics and free from apparent other diseases. Only 9 patients complete the 2 month treatments.

B-Patients: 19 patients aged 17- 47 years with AA (with no previous treatment) were included in this study. Treatment schedules included a combination of antioxidants [vitamin A (5000 I.U. /day), vitamin E (100 mg/day) and vitamin C (500 mg/day)].The treatment with nutrient antioxidants for patients with AA included in this study continued for two months.

C-Samples: heparinized venous blood samples were collected from patients with AA as well as from controls using plastic disposable syringes. Fresh blood samples were used for determination the level of malondialdehyde which is the by product of lipid peroxidation (MDA) and glutathione which is the main body defense mechanism (GSH) measurements in lymphocytes and erythrocytes (RBC) .Human lymphocytes were separated from fresh blood according to boyum method using lymphoprep solution(7) and frozen for up to 14 days for total antioxidant status(TAS) measurement using

commercial assay kit obtained from Randox(8). Small aliquots(0.5 ml) of patient samples were stored at – 20C0 until biochemical analysis performed.

D- Methods:

- Erythrocytes Malondialdehyde (MDA) Assay

Measurement of erythrocyte MDA (which is a by-product of lipid peroxidation), based on the reaction of thiobarbituric acid (TBA) forming TBA-MDA adduct, was carried out using the modified method of Stocks and Dormandy(9) as described by Gilbert et al(10). The results were expressed as OD /g Hb .A one-ml aliquot of ten-percent suspension of red cells in saline-azide was pre-incubated for 5 min at 37°C following 30 min. incubation at 37°C, the reaction was terminated by addition of 1.0 ml 28% (TCA-0.1M sodium arsenite). The mixture was centrifuged, 2.0 ml of supernatant was combined with 0.5 ml distilled water and 1.0 ml of 0.5% TBA in 0.05 M sodium hydroxide. Color development was achieved by boiling for 15 min. The tubes were cooled under tap water and the extent of MDA production was estimated from the absorbences at 532 and 453 nm (9) (10).

- Lymphocytes Malondialdehyde (MDA) Assay

To 0.25 ml of lymphocytes solution, a 0.75 ml saline-azide was added, the same procedure as that described for erythrocytes MDA was followed. The results were expressed as OD/ mg protein.

- Erythrocytes Glutathione (GSH) Assay

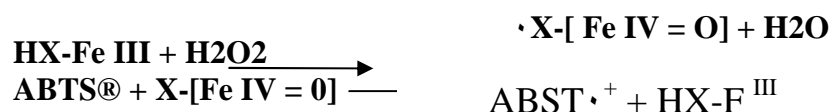
Erythrocytes GSH content was determined by the method of Godin et al. (11) Aliquots of 0.1 ml packed erythrocytes were used, combined with 0.1 ml distilled water and 0.65 ml of 5% TCA–1mM Na2EDTA. Then centrifuged at 3000 RPM (rotation per minute) for 5-8 min. at 4C0 and the supernatant was analyzed for sulphhydryl group content at 412 nm using 3 mM DTNB in phosphate buffer. The assay mixture contained 2.6 ml of 0.1 M phosphate buffer (pH = 8.0), 0.3 ml supernatant and 0.1 ml DTNB. The absorptivity of the solution at 412 nm was measured after 2 minutes waiting. Amounts of GSH were expressed as OD/g Hb.

- Lymphocytes Glutathione (GSH) Assay

Lymphocytes of about 0.5 ml were used to assay GSH with 0.5 ml 5% TCA –1mM Na2EDTA as described above. The results were expressed as OD/ mg protein for lymphocytes.

- Total antioxidant status (TAS)

Assay principle: ABTS® (2, 2-- Azino-di-[3- ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H2O2 to produce the ABTS® radical. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidant in the added sample causes suppression of this color production to a degree which is proportional to their concentration (8).



- **Plasma total bilirubin determination**

Plasma total bilirubin levels was determined based on the reaction of sulfanilic acid with sodium nitrite to form diazotized sulfanilic acid. In the presence of dimethyl sulfoxide, total bilirubin reacts with diazotized sulfanilic acid to form azobilirubin. In the absence of dimethyl sulfoxide only direct bilirubin reacts with diazotized sulfanilic acid to form azobilirubin (12) (13).

- **Serum Zn and Cu determination**

Serum Zn and Cu were measured by flame atomic absorption spectrophotometry [(F.A.A.S) Shimadzu AA-670/ GU-7]. Dilution of the serum was made by deionized water according to the sensitivity of the (F.A.A.S) and as mentioned in the manual instruction of the manufacturer in order to avoid the viscosity and to decrease the interference of the protein (14).

Results

A- Biochemical results

Table 1 shows oxidative stress indicator levels of the patients and their controls.

Statistical comparison between lymphocytes and erythrocytes oxidative stress indicator levels of the control and patients showed significant difference ($P < 0.05$). Treatment with antioxidants treatment significantly elevated body antioxidant defense content levels and decrease the level of oxidative stress by product (MDA) after 1 and 2 months of treatment compared to baseline values.

B- Clinical results

There is no increase in patient's body weight, no presence of acne, no gastrointestinal tract disturbance because we not use steroidal therapy (Table 2).

An obvious improvement in the rate of hair growth after 2 months treatments with antioxidant (picture 1).

10 patients depressed from the first month because of their slow hair growth, so they discontinue treatment and only 9 patient continuous 2 month treatments and they got complete hair growth.

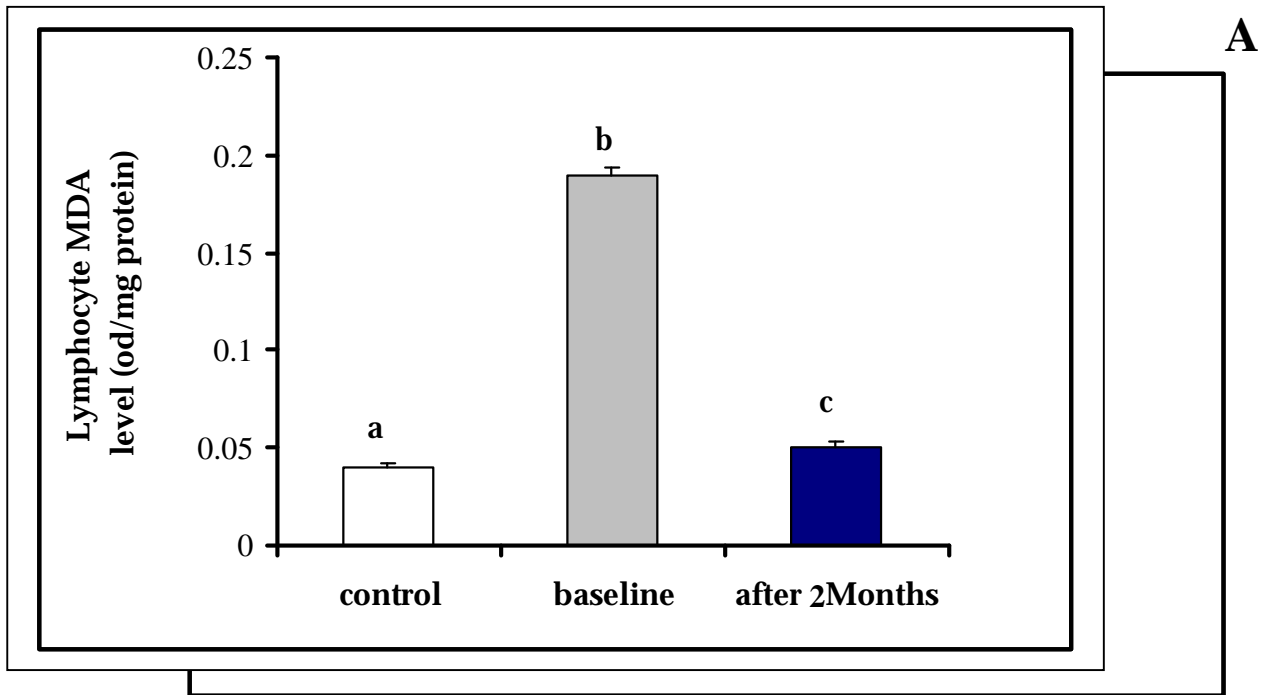
Table (1): Markers of oxidative stress in patients with alopecia areata and their controls before and after two months treatment with a combination of antioxidants

variables	Controls N= 25	Patients with alopecia areata	
		Months after treatment	
		0 N=19	2 N=9
Lymphocytes MDA (OD/mg protein)	0.04 ± 0.002 a	0.19 ± 0.004 b	0.05 ± 0.003 c
Erythrocytes MDA (OD/g Hb)	0.22 ± 0.01 a	0.92 ± 0.021 b	0.15 ± 0.013 c
Lymphocytes GSH (OD/ mg protein)	1.62 ± 0.044 a	0.79 ± 0.03 b	1.38 ± 0.042 c
Erythrocytes GSH (OD/g Hb)	20.3 ± 0.98 a	10.0 ± 0.52 b	19.78 ± 0.51 c
Lymphocytes TAS (umol/mg protein)	1.17 ± 0.095 a	0.51 ± 0.03 b	1.52 ± 0.09 c
Plasma TSA (mmol/L)	1.58 ± 0.58 a	0.42 ± 0.01 b	1.50 ± 0.013 a
Plasma total bilirubin (umol/L)	7.29 ± 2.35 a	5.99 ± 1.8 b	6.92 ± 2.70 a
Serum zinc (ug/dl)	98.2 ± 0.87 a	66.35 ± 5.30 b	75.03 ± 6.82 c
Serum copper (ug/dl)	81.3 ± 1.71 a	90.35 ± 5.11 b	77.82 ± 2.5 c

Values are expressed as mean ± standard deviation.

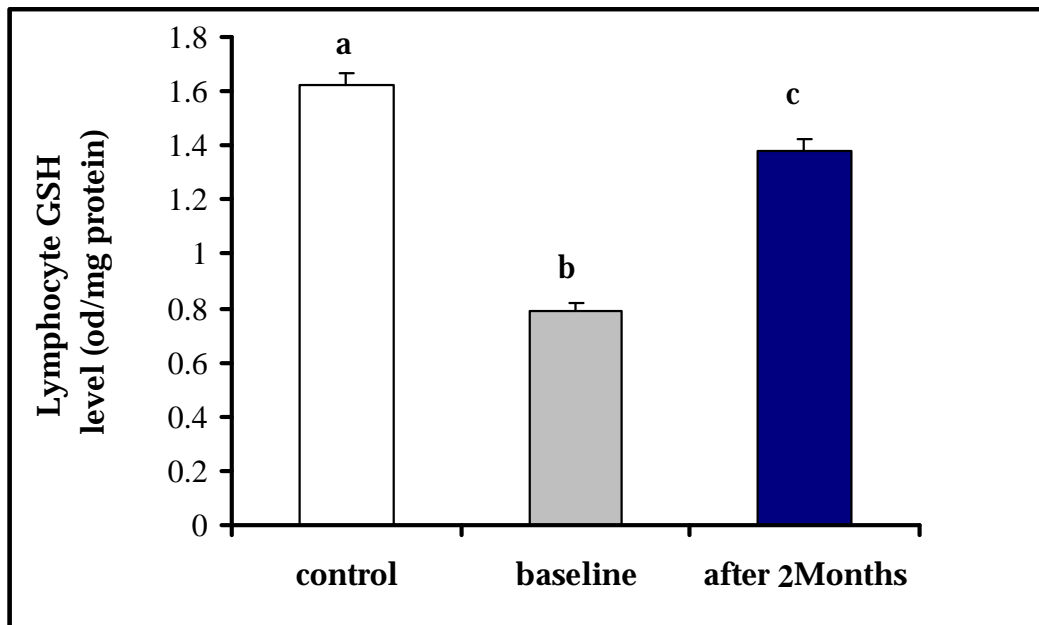
Values with non-identical superscripts (a, b, c) within the same parameter considered significantly different, P<0.05.

N=number of subject

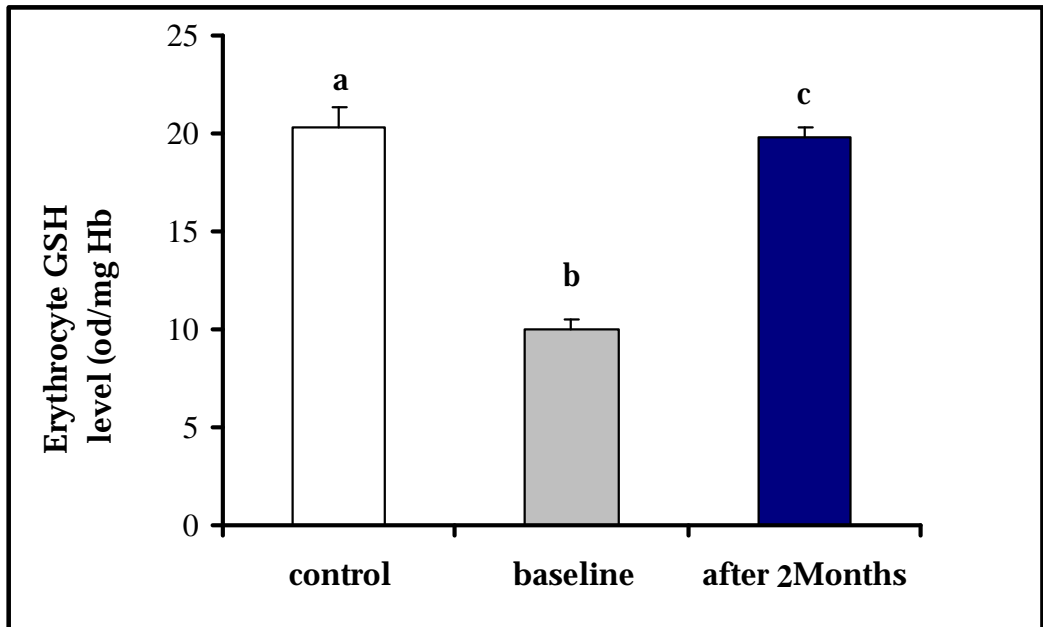


B

Figure (1): Malondialdehyde level (A) in lymphocytes (B) in erythrocytes of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants

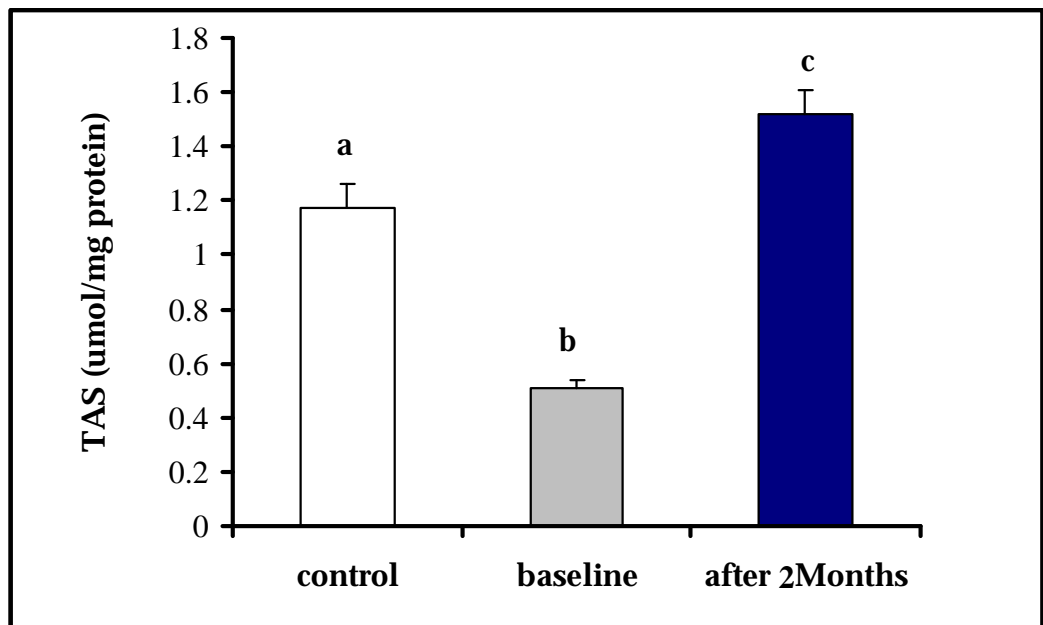


A

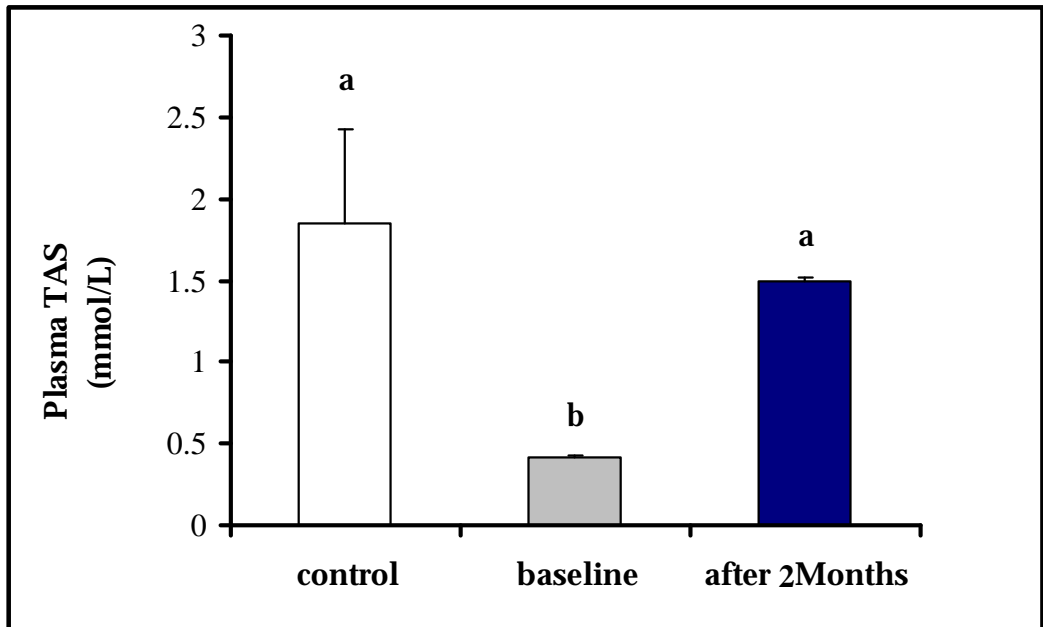


B

Figure (2): Glutathione level (A) in lymphocytes (B) in erythrocytes of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants



A



B

Figure (3): Total antioxidant status level (A) in lymphocytes (B) in plasma of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants

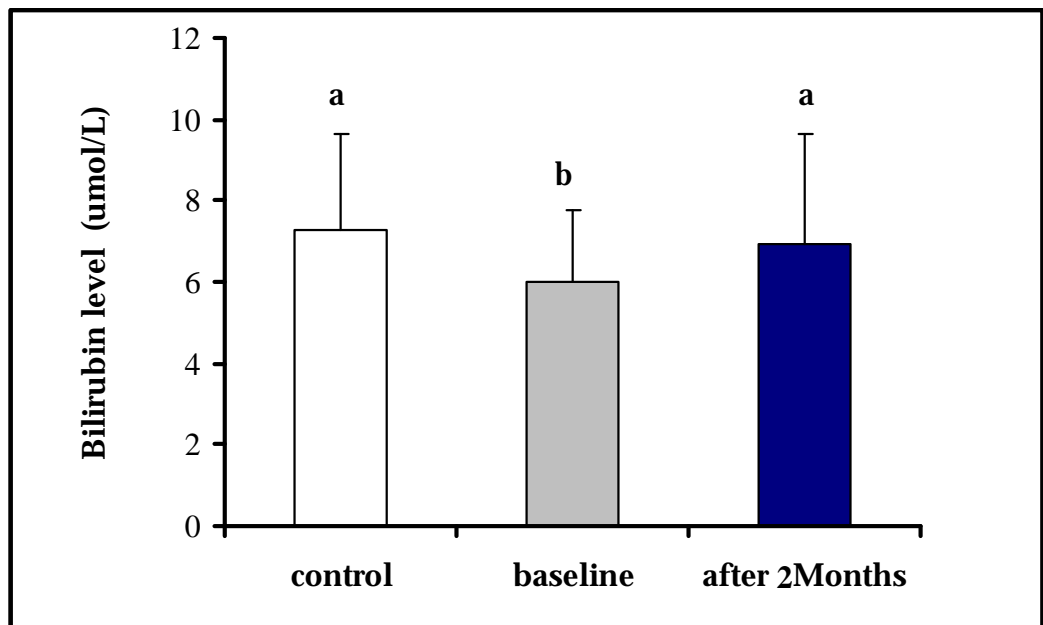


Figure (4): Plasma total bilirubin level of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants

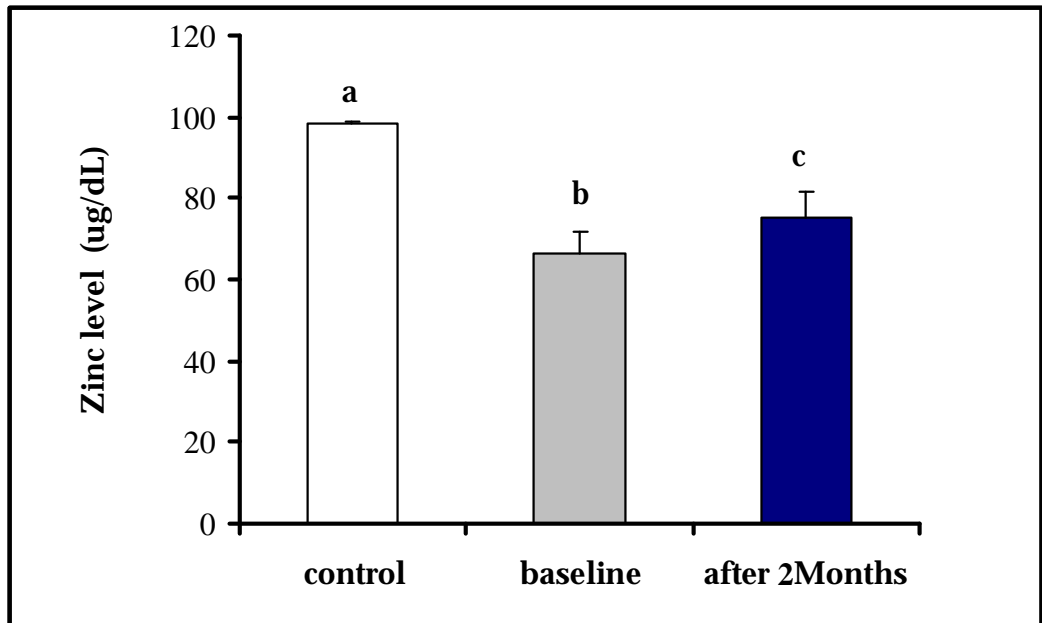


Figure (5): Serum zinc level of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants

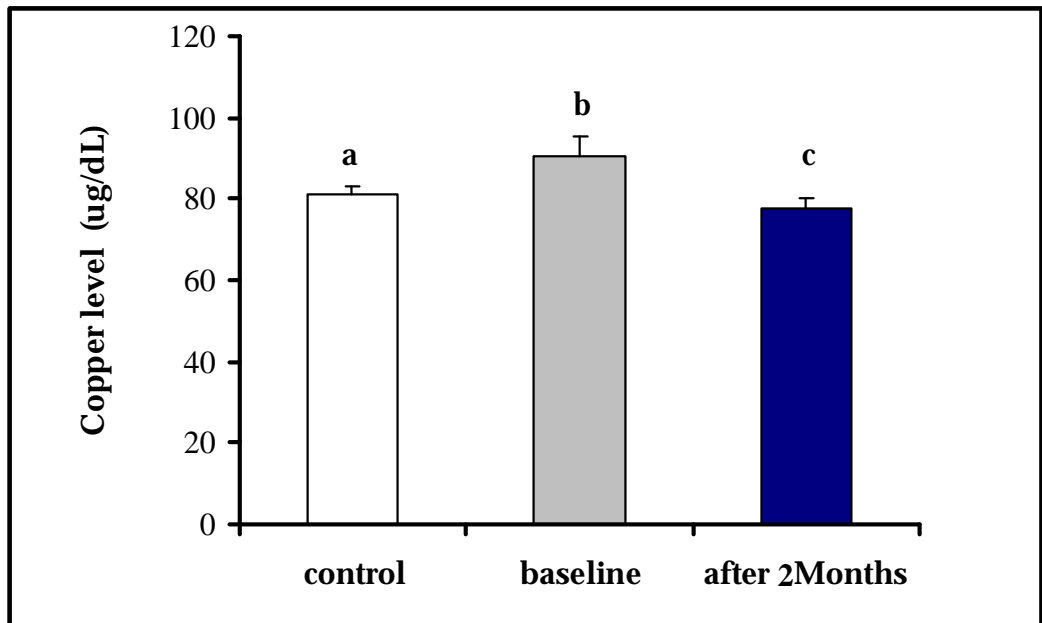


Figure (6): Serum copper level of healthy controls and patients with alopecia areata before (baseline) and after 2 months treatment with combination of antioxidants

Table (2): Clinical results of patients with alopecia areata with their aged matched controls before and after two months treatment with a combination of antioxidants

Clinical sign	Controls N=25	Patients with alopecia areata	
		Pre-treatment N=19	After two months treatment N=9
Body weight (Kg)	72.35±15.99	70.59±15.63	70.29±15.30
Presence of acne	negative	negative	negative
Presence of gastrointestinal disturbance	negative	negative	negative
Depression	negative	10 patients positive 9 patients negative	negative

Values are expressed as means ± SD.

* Significantly different from control (p< 0.05).

† Significantly different from pretreatment values (p<0.05).

N Number of subjects

Severity of the presence of acne and gastrointestinal disturbance determined by dermatologists.



A



B

Patient (1): O.N 25 Year's old male with alopecia areata with no previous treatment
A: before treatment, B: after two month treatment with antioxidants.

Discussion

This study showed alterations in oxygen free radical scavenging process in lymphocytes and erythrocytes of patients with AA manifested by decreases in lymphocytes and plasma total antioxidant status, lymphocytes and erythrocytes GSH and

an increase in their basal MDA level, decrease in plasma total protein, decrease in serum Zn and increase in serum copper level. It seems that antioxidant defenses remain high in normal individual, keeping lipid peroxidation under control. However, in patients with AA antioxidant defenses are significantly lowered, thus exposing lymphocytes and erythrocytes to the damaging effects of reactive oxygen species (ROS).

The data of this study revealed that the total endogenous basal MDA levels in both lymphocytes and erythrocytes of patients with AA were significantly higher than those of controls (Table & Figure 1). This may be due to the direct and indirect effect of alopecia, per se, where the already formed immune complex, has resulted in the production of phagocytes-derived free radicals. As a consequence, lipid peroxidation occurred which was demonstrated by the significant increase in total endogenous basal MDA levels.

GSH, a major scavenger of oxygen reactive intermediates, protect cells against the effect of free radicals and of related intermediates (e.g. peroxides) that are formed endogenously (15). Therefore, a reduction in cellular GSH may be associated with an increased susceptibility to oxidant stress or reflect a response to it (16). In the present study, the total lymphocyte GSH content in patients decreased to 48.77 % in cases of that of the total lymphocytes content of healthy controls (Table 1 & Figure 2). On the other hand, GSH content in erythrocytes decreased to 49.26 % in cases of the controls values (Table 1 & Figure 2). This may be due to the increased GSH utilization in neutralizing the phagocytes derived free radicals which can damage both the source cell and cells in close apposition to stimulated phagocytes. These changes in GSH level may increase the susceptibility of lymphocytes and erythrocytes to endogenous oxidative stress. A considerable decrease in the erythrocytes GSH has already been reported in patients with alopecia (17).

Total antioxidant status (TAS) is an important marker of disease since ROS circulate freely in the body, and can have a serious repercussion throughout the body. The body utilizes antioxidant reserves to cope with free radicals, and monitoring antioxidants levels may be conducive to the early detection of disease. Therefore, a complete antioxidant profile may ensure accurate detection of variations in antioxidant levels caused by disease onset.

Lymphocytes and plasma from patients with AA showed a lower TAS levels than the controls (Table 1 & Figure 3), suggesting the presence of a generalized decrease in antioxidant status in the patients which may be attributed to the utilization of body antioxidants in neutralizing the increased endogenous free radicals. The antioxidant defense system has many components. A deficiency in any of these components can cause a reduction in the overall antioxidant status of an individual. Total antioxidant status enables assessment of the integrated antioxidant system which encompasses all biological components with antioxidant activity. Reduction in total antioxidant status has been implicated in several disease states, such as cancer and heart disease and patients with poor nutritional status (18) (19).

Bilirubin is one of the natural defenses against free radicals; it has ability to protect albumin bound polyunsaturated fatty acid (20). The decrease serum bilirubin levels in patients with AA (Table 1 & Figure 4) could be attributed to the increased utilization of bilirubin in neutralizing phagocytes-derived free radicals. It is unclear, at the present time, the exact mechanism behind the protective effect of combination therapy on serum bilirubin.

Zinc, an essential trace element, is important in numerous critical biochemical processes since it's a cofactor in about 200 metalloenzymes including Cu/Zn- superoxide dismutase, a critical cytoplasmic antioxidant enzyme (21). Since Zn is an essential

component of Cu, Zn-SOD, the deficiency of Zn could induce an increase in tissue oxidative damage.

The present study showed a significant decline in serum Zn level in patients with AA (Table 1 & Figure 5), a fact that agrees with that of Tasaki et al (22). Factors responsible for this decline in Zn levels are unknown. However, decreases in plasma Zn content has been attributed to reductions in intake or absorption from small intestine or to increases in urinary loss, or to redistribution from plasma to tissue (23). Furthermore, tissues with high cellular turnover (e.g. skin) are characteristically affected by Zn deficiency, calling the attention to the possibility that some dermatological manifestations, such as AA, may be attributed to Zn deficiency (24).

Copper could be a potential inducer of LDL oxidation. On one hand, Cu has the ability to oxidize LDL in-vitro (25). On the other hand, it is a constituent of Cu, Zn-SOD which is involved in preventing oxidative injury. In addition, caeruloplasmin, a multifunctional protein which contains most of the Cu in blood, is thought to possess antioxidant functions, which could be beneficial in resisting disease. In contrast, high caeruloplasmin levels have been speculated to be a risk factor for atherosclerosis, based on its pro-oxidant properties (26).

A serum Cu level was significantly higher in patients with AA as compared with their aged matched controls (Table 1 & Figure 6). This may be attributed to zinc deficiency which is associated with an increase in Cu due to the antagonistic relationships between them, since Zinc will compete for copper for receptors involved in absorption (compete for sulfhydryl binding sites)(27) . Other investigation suggested that serum copper concentration raised in response to activation of interleukin-1 during the acute phase reaction (28). Interleukin-1 caused an elevation of serum copper concentration and decreased serum zinc concentration, and this may be the case in AA. Increased levels of Cu may also be attributed to a rise in copper-binding capacity of caeruloplasmin (29). However, the present finding does not agree with that of Tasaki et al (22) who failed to observe an elevation in Cu concentrations in patients with AA

Effect of treatment with a combination of antioxidants:

The results of the present study clearly demonstrated that the ability of antioxidants to correct most of the observed alterations in antioxidant defense mechanisms in patients with AA.

In this study, patients with AA were treated with a combination of antioxidants which include, vitamin A, C and E. These antioxidants were found to decrease the basal level of MDA .Optimum effect of treatment was obtained after 2 months. This could be due to the fact that supplementation with vitamin A, C and E may elevate these antioxidants to levels that successfully attenuate the increased oxidative insult.

The antioxidant treatment, led to increase lymphocytes and erythrocytes GSH contents and the levels of lymphocytes and plasma total antioxidant status. These effects may be due to a direct and/or an indirect scavenging activity of these vitamin antioxidants, a fact that may in turn lead to inhibition the oxidation of protein and decrease the utilization and damage of GSH and proteins by free radicals.

The combination therapy affects positively the immune system. The traditional treatment (especially prednisolone) affects the immune system by decreasing the immune complex deposition, while the nutrient antioxidants may act by inhibiting the negative effect of ROS on immune system, thus increasing rate of hair growth. The mechanisms by which these vitamins protect the immune system could be through either a direct and/ or an indirect effect. Vitamin A or retinol is essential for innate (non-specific) immunity.

Semba noted that Vitamin A is an immune enhancer that has been shown to increase lymphocyte clonal proliferation responses to antigens and mitogens, increase antibody responses to T cell-dependent antigens, inhibit programmed cell death (apoptosis), and restore the health and function of damaged mucous membranes (30). Supplementation with vitamin E leads to reinforce the immune system's resistance to any infectious disease (31). The indirect effect may be through increasing the vitamin A level in body since high dose of corticosterone resulted in a rapid loss of vitamin A from plasma, liver, adrenals and thymus. Vitamin C is an "immunostimulatory, anti-inflammatory, anti-allergic" vitamin (32). Anderson also noted that many experiments showed vitamin C to enhance T lymphocyte reactivity to mitogens in humans and animals. The indirect effect is that vitamin C is a powerful antioxidants that protect against damaging effect of phagocytes derived radicals so patients using corticosterone for periods longer than 2 weeks should take vitamin C supplement to counteract the depleting effect of corticosterone since the steroidal anti-inflammatory drugs because increased loss of vitamin C through the urine. Vitamin E (The antioxidant and immunostimulant) and vitamin C act synergistically to protect membranes from lipid auto-oxidation and play a key role in protecting phagocytes from damage by self-generated free radicals, since immune cells have a high percentage of easily oxidized fatty acids in their membranes (33). Phagocyte membrane auto-oxidation is a major immune problem, since macrophages are necessary to antigenically activate T lymphocytes. The indirect effect of vitamin E include elevating the level of the antioxidant (vitamin E) which is depleted by the high dose of corticosteroid, and hence increase body defense mechanism against free radicals.

The ability of the combination therapy to elevate bilirubin, levels are suggestive of either a direct effect through scavenging endogenous ROS, or an indirect effect by increasing the activity of endogenous antioxidants. Either probability or both may in turn lead to an inhibition of the mechanisms leading to initiation of lipid peroxidation and consequently oxidative stress. However, it is unclear, at the present time, the exact mechanisms behind the protective effects of this combination therapy on bilirubin. Furthermore, there is no comparable report in the literature concerning the effects of vitamins antioxidants therapy on bilirubin, uric acid and protein metabolism in alopecia areata.

Vitamins therapy significantly increased Zn levels and decreased Cu levels and corrected the imbalance in these trace metals metabolism in alopecia. Such an imbalance was reflected in the Zn/Cu ratio, which was decreased in patients with AA compared with controls (data not shown). The mechanism responsible for this improvement in Zn/Cu ratio, as well as its significance, is unknown at present. Tasaki et al (22) demonstrated that Zn/Cu ratio clearly reflects the severity of the progression of skin diseases. Clearly, further studies are required to investigate this hypothesis and to investigate whether or not it may be useful in assessing the effects of various therapies, including vitamins antioxidants against AA. Whether or not vitamins antioxidants corrected this ratio through their effects on intake, absorption or redistribution of these trace metals, especially Zn, needs to be investigated.

The most important point in the clinical significance of antioxidants is in improving the hair growth response of patients with AA. This finding, which is reported for the first time, may be due to the powerful scavenging activity of these antioxidants, which in turn may decrease damaging and utilization of GSH in neutralizing the phagocytes- induced free radicals. So replenishment of GSH within natural killer (immune) cells may strengthen the immune system and increase the rate of hair growth.

In conclusion, the protective effect of vitamins combinations on tissue antioxidant status, in addition to the clinical improvement in the rate of hair growth, suggests the use of antioxidants treatment for the management of patients with AA.

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