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RESEARCH ARTICLE

Role of Vitamin B12, Folic Acid and Oxidative Damages, in Serum of Patients with Vitiligo

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Abstract

Background: Vitiligo is a chronic common disease which may cause as a result of oxidative stress which is proposed to implicate in its etiopathogenesis. Vitiligo is an autoimmune disease affecting skin, hair and oral mucosa. This disease is characterized by a loss of melanin. Disturbance of thyroid hormones has been known as a key trigger of this pathology. Objective: The aim of this study is to determine the levels of vitamin B12, folic acid , oxidative stress (lipid peroxidation ;MDA),total antioxidant capacity (TAC), Paraoxanase (PON1)in serum of patients with vitiligo compared to matches control group. Materials and subjects: The study included 42 patients from Egyptian population suffering from generalized vitiligo (29 male patients and 13 female patients; mean age 35.1 years; ranging from 28-45 years and 36 healthy volunteers as control group (22 male patients and 14 female patients; mean age 36.2 years; ranging from 25–47years). Vitiligo patients were recruited from Medical research center of excellence of National Research Centre, Egypt, during interval of 6 months (1 October, 2018 to 31 March , 2019). Vitiligo diagnosis is based on if areas of patient's skin, hair or eyes lose coloring and confirmation is done by using Wood's lamp. Blood samples were obtained from the patients and controls. PON1, MDA, TAC vitamin B12 (vit.B12) and folic acid biomarkers were analyzed in serum samples of both control and vitiligo patients. Results : The present results declared significant elevation of oxidative stress ; MDA (+159.26), which is associated with significant decrease in PON(-34.10) ,TAC (-35.89),vitamin B12(-53.41) and folic acid (-89.23) in vitiligo patients compared to control group. Conclusion: Oxidative stress and antioxidant balance are strongly implicated in the vitiligo patients .Vitiligo disease should be examined for multifactorial such as homocysteine levels for a possible implication due to the postulation in the pathogenesis of vitiligo based on the previous findings . Moreover, the therapeutic role of supplementation with Vitamin B12/folic acid in vitiligo needs to be studied further.

Keywords: *Autoimmune disease, Melanin loss, Oxidative stress, Vitiligo, Vit. B12, Folic acid, TAC,PON.*

Introduction

Vitiligo is a disorder included genetic and autoimmune skin disease in which skin loses its natural colour due to pigmentation lack. Vitiligo usually develops before age 40, and people suffering with this disorder develop white and irregularly shaped patches on different skin areas. It can change the colour of hair and eyes, and white patches can also appear inside the mouth. Although, the exact etiology of vitiligo is still largely unknown, destruction of melanocytes, cells that produce melanin, a dark pigment responsible for skin colour, by the body's own immune system is considered to be the main cause of vitiligo [1,2]. 5Although, the exact etiology of vitiligo is still largely unknown, destruction of melanocytes, which are melanin producing cells, a dark pigment responsible for skin color, by the body's own immune system is

considered to be the main cause of vitiligo [3,4].

Moreover, vitiligo may co-occur with other autoimmune disorders, such as hypo-or hyper-thyroidism, diabetes, adrenocortical insufficiency, rheumatoid arthritis, and pernicious anemia. Other triggering factors include sunburn, environmental or industrial chemicals, and stress [5-7]. Studies have identified that NALP1, a protein coding gene that encodes a protein related to apoptosis, plays an important role in developing vitiligo. However, despite having a genetic linkage, only 57% of children are likely to get vitiligo even if one of their parents has it. Vitiligo is neither contagious nor life threatening. However, the condition is often stressful, and people may undergo serious depression in some of the worst cases [4].

Vitamins are known to play important roles in the process of skin Pigmentation. Vitamin B12 inhibits the production of homocysteine, a homologue of amino acid cysteine. Homocysteine down regulates the activity of tyrosinase, an enzyme responsible for melanin production, as well as generates free radicals, leading to impaired melanin synthesis and destruction of melanocytes. In this whole process, folic acid works in tandem with vitamin B12 as a methyl group donor. This is why it is always recommended to take these two vitamins together in order to treat vitiligo. According to some scientific studies, a combination of vitamin B12 and folic acid supplementation and sun exposure is a good strategy to regain natural skin color [2,4,8].

Oxidative stress was first recognized in vitiligo by the existence of high levels of H2O2 in the diseased skin, and disturbance in balance of reactive oxygen species (ROS) in blood and tissues of vitiligo patients, particularly in active disease [9]. ROS can destruct key lipid, protein, and enzymes involved in melanogenesis, and also impair protein-repair mechanisms [2]. In addition, there is also evidence of deficient antioxidants [10]. Paraoxonase (PON) family of antioxidant enzymes destroy oxidized phospholipids, and sometimes defective in various disease situations. Polymorphisms of PON gene are linked to metabolic syndrome [11]. The goal of study is to investigate the role and serum levels of vitamin B12, folic acid, PON1, oxidative stress; MDA and TAC in Egyptian patients with active generalized vitiligo, in addition to controls.

Materials and Subjects

Subjects

The study included 42 patients from Egyptian population suffering from generalized vitiligo (29 male patients and 13 female patients; mean age 35.1 years; ranging from 28–45 years and 36 healthy volunteers as control group (22 male patients and 14 female patients; mean age 36.2 years; ranging from 25–47years).

General dermatologic examination was done. The site of lesions was documented. Subtypes of vitiligo were demarcated clinically as segmental or non-segmental. Disease severity and degree were measured using the palmar unit measurement (one palmar unit signifies the depigmented lesion dimensions of the palm of the hand). Depending on history delivered by the patients, Vitiligo Disease Activity Score (VIDA) was used to detect whether the disease is active or stable. Active disease denotes an increase of lesions with emergence of novel lesions in the preceding year. Vitiligo patients were recruited from Medical research center of excellence of National Research Centre, Egypt, during interval of 6 months (1 October, 2018 to 31 March, 2019). The mean period of the disease in the vitiligo group was 6.7 years. Vitiligo diagnosis is based on if areas of patient's skin, hair or eyes lose colouring and confirmation is done by using Wood's lamp.

Blood samples were obtained from the patients and controls. PON1, MDA, TAC vitamin B12 and folic acid biomarkers were analyzed in serum samples. The study protocol followed the Declaration of Medical Division, National Research Centre (NRC) ; Participants were informed about the study protocol, in addition to written consent which was gathered from them. Study was permitted at the Ethics Committee of NRC, Cairo, Egypt with no" 18-167.

Active disease denotes an increase of lesions with emergence of novel lesions in the preceding 6 months. Stable disease indicates lack of change in vitiligo lesions in the 6 months preceding the study as viewed by patient. Progressive disease – indicates increase of formerly present lesions and/or

the emergence of new lesions in the 6 months preceding the study as viewed by patient. Regressive disease – indicates improvement of lesions either spontaneous or after ultraviolet treatment in the 6 months preceding the study. Degree of vitiligo was measured by the Vitiligo Area Scoring Index (VASI) [12]. Equivalent to the Psoriasis Area Severity Index used for psoriasis. The total body VASI was then calculated using the following formula by considering the contribution of all body areas (possible range, 0–100):

VASI = Σall body sites (hand units) × (residual depigmentation).

The activity of vitiligo was measured by vitiligo Disease Activity score (VIDA score) [13]. (Table 1).

Exclusion Criteria

Exclusion criteria comprised the presence of chronic disease; concurrent inflammatory disorder, such as infections; immuno-compromised state; diabetes mellitus; malignant disorders; liver or kidney disorders; and recent principal surgical operation. Examination of untreated patients having active lesions of generalized vitiligo was done [14].Patients with segmental vitiligo was omitted. Patients receiving antioxidant drugs, vitamins, or hormone replacement treatment, in addition to smokers and patients suffering from alcoholism were also omitted.

Inclusion Criteria

Inclusion criteria comprised the following: age＞18 years old; both sexes; not pregnant; nonsmoker; no history of autoimmune disorder, concurrent dermatological disorder, or thyroid dysfunction; alcohol intake, vitamin intake, or using drugs such as anti-inflammatory medications. Furthermore, all patients did not receive any treatment for 3 months preceding the study.

Laboratory Analysis

Sample Collection and Preparation

All participants did not receive any medications for at least 24 h before blood collection. Clinical and routine biochemical parameters were calibrated using basic protocols. Peripheral blood specimens from vitiligo patients and clinically healthy individuals were gathered from Dermatology Dep., Excellence Centre Clinic, NRC, Egypt. Blood specimens were collected in EDTA-containing tubes and anticoagulant-free tubes after an overnight fast. After immediate centrifugation (3,000 g) for 10 min at 4oC, plasma and serum were separated in Eppendorf tubes and frozen immediately at −80oC until analysis.

Serum Vitamin B12 and Folic Acid

Serum folate and vitamin B12 levels were determined by enzyme-linked immunosorbent assay using a Unicell DXI 800 (Beckman Coulter) autoanalyzer.

Measurement of Oxidative Stress

MDA, (produced from lipid peroxidation), was calibrated by the TBARS technique[15]. in which MDA reacts with TBA (Thiobarbituric acid) at 90-100°C, pH=2-3, within 15 minutes producing a pink pigment. One volume of the specimen was assorted with two volumes of the solution containing 15% tri-chloroacetic acid (w/v), 0.375% TBA (w/v) and 0.25 N hydrochloric acid, and the mix was positioned in boiling water bath for 30 minutes. After cooling, specimens were centrifuged once more, at 3000 rpm for 15 min, and the absorption was read at 532 nm by the spectrophotometer. TBARS was ultimately calculated with regard to the molar absorption coefficient of 1.56×105 M-1cm-1.

Measurement of Paraoxonase 1 (PON1) Activity

PON1 activity was measured using paraoxon substrate (Sigma Chemical Company, St Louis, MO, USA). The rate of paraoxon hydrolysis (diethyl-p-nitrophenyl phosphate) was calibrated by supervising the increase of absorbance at 412 nm, at 37°C. The quantity of created p-nitrophenol was determined from the molar absorption coefficient at pH 8, which was 17,000 M−1 cm−1. PON1activity was expressed as U/l serum[16].

Measurement of Total Antioxidant Capacity

TAC levels of serum were detected using commercial colometric assay diagnostic kits (Biodiagnostic, Cairo, Egypt). Fe2+-o-dianisidine compound with hydrogen peroxide by Fenton-type reaction originates to OH radicals. The reduced ROS interacts with colourless o-dianisidine molecules in low

pH to produce yellow-brown dianisidine radicals. Dianisidine radicals increase the creation of the colour by contributing in an advanced oxidation reaction. Though, antioxidants which halt these oxidation reactions overwhelm the formation of the colour. Outcomes are offered by automated analyzers used to caliberate this reaction spectrophotometrically at 240 nm. Trolox (water-soluble vitamin E analogue), is used as a calibrator. Outcomes were expressed as micromoles Trolox. Equivalent per litre (µmol TE/L) [17].

Table 1: Demographic and clinical characteristics of vitiligo patients and controls

Characteristic	Vitiligo (n=40)	Control (n=36)	*p*-value
Age (y)	36.87±11.09 (35.1)	38.00±4.81 (36.2)	< 0.001
Sex (female/male)	29/13	14/22	0.01
Subtypes(segmental/ non-segmental	30/40	-	
VASI	5.00±1.20	-	
VIDA (active/stable)	20/23	-	
Period of disease (y)	6.74±0.80	-	

Values are displayed as mean±standard deviation (median). VASI: Vitiligo Area Scoring Index, VIDA: Vitiligo Disease Activity Score.

Results

Significant reduction in vitamin B12 and folic acid reached to 53.41 and 89.23 %, respectively compared to control value (Table 2) was detected. Additionally, Table (3), revealed marked decrease in PON 1 and TAC with percentages 34.10 and 35.89%, respectively, while significant increase in MDA with percentages 159.26% compared to matches control.

Table 2: Vitamin B12 and Folic acid levels in vitiligo patients compared to matches control

Markers	Vitiligo subject	Control Subject
Vitamin B12(Pg⁄ml) %Change	186 ±11.50^{h} -53.41	399. 23 ±19.00^{g}
Folic acid(ng⁄ml) %Change	1.22±0.20^{j} -89.23	11.33±0.67^{i}

Data are displayed as Mean ±SD . Statistical analysis is carried out using SPSS computer program version 8 combined with co-state computer program , where different letters are significant at p ≤0.05.

Table 3: PON1 activity and oxidative stress markers in vitiligo patients compared to matches control

Markers	Vitiligo Subject	Control Subject
MDA (*u* mole /L) % change	6.30± 0.38^{f} +159.26	2.43± 0.01^{e}
TAC(TAC (µmol Trolox Eq./L) % change	0.75±0.07^{c} -35.89	1.17±0.20^{d}
PON1 (U/L) % change	100.23±9.43^{b} -34.10	152.00 ±11.00^{a}

Data are expressed as Mean ±SD . Statistical analysis is carried out using SPSS computer program version 8 combined with co-state computer program , where different letters are significant at p ≤0.05.

Discussion

The current study declared significant increase in oxidative stress biomarkers; MDA, while marked reduction in TAC and PON1 levels in vitiligo patients compared to matches control. One of the chief theories in pathogenesis of vitiligo is oxidative stress hypothesis [9]. Which is cantered on the production of some toxic metabolites during the biosynthesis of melanin, resulting in hydrogen peroxide causing melanocytes destruction through inhibition of the activity of CAT. Disturbance in oxidant/ antioxidant

balance concomitant with accumulation of hydrogen peroxide and low concentration of CAT in epidermis and serum of vitiligo patients[2,9]. Endogenous ROS resulting from epidermal sources, for example radicals produced as a result of activation of neutrophils or enzymes, as lipooxigenase; moreover it can be exogenous as prooxidative stimulants; microorganisms, pollution, and xenobiotics[18].

The balance between the systems of antioxidant enzyme and oxidative damage suggesting the physiological and pathological effects of ROS[3]. These stimulants interact with biological molecules causing damaging effects in biologic systems .Oxidative damage was initiated during the biosynthesis of melanin due to the production of 3,4-dihydroxyphenylalanine, dopachrome, and dihydroxyindole which are considered as intermediate products and have strongly direct toxic effects on melanocytes[4].

Further, destruction of melanocyte takes place in vitiligo patients as a result of formation of toxic H2O2 compound and inhibiting the process of detoxification by stopping the activity of CAT, resulting in oxidative stress [2,19]. The skin antioxidant system involves network of an enzymatic and non-enzymatic antioxidants. The glutathione peroxidase, CAT, and superoxide dismutase are considered as a system of enzymatic antioxidant while, α-tocopherol, ubiquinone, ß-carotene, ascorbate, and glutathione are non-enzymatic intracellular antioxidants [20].

ROS are elaborated in healthy individuals as a result of normal metabolism, and is eliminated by the defense of antioxidants mechanism. So, oxidative stress occurs when an imbalance towards the side of oxidant occurs [21]. In this study, vitiligo patients showed reduced serum levels of TAC. This denotes disturbance in oxidant/antioxidant balance in serum of vitiligo patients, providing support for free-radical-mediated damage in vitiligo.

Yesilova et. al. [2] found that serum antioxidants were lower ,while serum oxidants was higher, in vitiligo patients in contrast to healthy subjects, suggesting that balance of oxidative/ antioxidative is moved towards the oxidative side.

Also, significant inhibition in PON1 activity was detected in vitiligo disease in the present results. Preceding studies have declared ROS role in pathogenesis of vitiligo and reduction of PON1 activity in oxidative stress as inflammatory cases like Behçet's disease [22].

PON1, is an enzyme correlated to HDL showing an anti-atherosclerotic function by preventing oxidation of LDL. Low activity of PON1 was documented to be attributed to dyslipidaemia, diabetes mellitus, increased oxidative stress. etc. In concomitant with the present results, it has been ascertained that the activity of serum PON1 inhibited significantly, and the levels of lipid peroxidase elevated significantly in vitiligo and metabolic syndrome patients [2,22]. The reduced concentration of PON1caused increased peroxidation of lipoprotein and oxidation of LDL -c.

Regarding Vitamin B12 and folic acid, only few studies have evaluated role of Vitamin B12, folic acid, and homocysteine in vitiligo. In the present study, we attempted to analyze the role of vitamin B12 and folic acid, in pathogenesis of vitiligo. Serum vitamin B12 and folate levels were noted to be significantly reduced in vitiligo patients in comparison to control.

Together vitamin B12 and folic acid are needed as cofactors for homocysteine methyl transferase enzyme for regeneration of methionine from homocysteine in the activated methyl cycle[4]. So, the deficiency of nutritional status in any of these two vitamins will result in homocysteine elevation and reduction in levels of methionine in the circulation.

The results obtained were in accordance with a recent Indian study done by Singh et al. [23],who reported that the mean values of vitamin B12 and folate were significantly decreased in vitiligo group as compared to control. VASI score is the involvement of serum albumin by vitiligo and the amount of depigmentation. The chronicity of vitiligo is produced as results of injury of melanocyte, either due to the drug course of disease or treatment lack, which shows an extensive depigmentation (greater VASI score).

Agarwal et al. [4], reported no association between folate, or vitamin B12 levels and the extent of vitiligo. Also, the relation between vitamin B12, folate levels, and VIDA score of patients was not statistically significant. This finding was consistent with the studies of Kim et al. [24] and Karadag et al. [25]. No significant association between the homocysteine, folic acid, or vitamin B12 level and patients dietary habits was observed by Agarwal et al. [4]. This is in accordance with previous studies which found no significant difference in serum homocysteine, folic acid, or vitamin B12 levels with different dietary habits [26].Dietary habit may affect level of homocysteine because vitamin B12 is mainly present in proteins of animal.

Conclusion

Oxidative stress and antioxidant balance are strongly implicated in the vitiligo patients which are a chronic and widespread disease. Vitiligo disease should be investigated for several factors such as serum homocysteine levels for a possible role of raised concentration due to the postulation in the pathogenesis of vitiligo based on the previous findings. Moreover, the therapeutic role of supplementation with vitamin B12/folic acid in vitiligo needs further studies.

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