PUBLICATIONS AND PRESENTATIONS

Publications

1. Shajil, E.M., Laddha, N.C., Chatterjee, S., Amina, R.G., Malek, R.A., Shah, B.J., and Begum, R. (2007). Association of *Catalase* T/C exon 9 and *Glutathione peroxidase* codon 200 polymorphisms in relation to their activities and oxidative stress with vitiligo susceptibility in Gujarat population. Pigment Cell Res. (In Press).

2. Shajil, E.M., Marfatia, Y.S., and Begum, R. (2006). Acetylcholine esterase levels in different clinical types of vitiligo in Baroda, Gujarat. Ind. J. Dermatol. *51*, 289-291.

3. Shajil, E.M., Agrawal, D., Vagadia, K., Marfatia, Y.S., and Begum, R. (2006) Vitiligo: Clinical profiles in Vadodara, Gujarat. Ind. J. Dermatol. *51*, 100-104.

4. Shajil, E.M., Chatterjee, S., Agrawal, D., Bagchi, T., and Begum, R. (2006). Vitiligo: pathomechanisms and genetic polymorphism of susceptible genes. Ind. J. Exp. Biol. *44*, 526-539.

5. Shajil, E.M., and Begum. R. (2006). Antioxidant status of segmental and non-segmental vitiligo. Pigment Cell Res. *19*, 179-180.

6. Agrawal, D., Shajil, E.M., Marfatia, Y.S., and Begum, R. (2004). Study of the antioxidant status of vitiligo patients of different age groups in Baroda. Pigment Cell Res. *17*, 289-94.

Presentations

1. Shajil, E.M., and Begum, R. (2005). Antioxidant status of different clinical types of vitiligo in Baroda. Poster presented at Two day TIFR seminar on modern biology "Facets and prospects" held on 10-11, October at Maharaja Sayajirao University of Baroda, Vadodara.

2. Shajil, E.M., Sheril, A., Sahani, M.H., and Begum, R. (2005). Evaluation of oxidative stress, autoimmune and neurochemical hypotheses in Baroda Vitiligo patients. Poster presentation at the First Conference of Asian Society for Pigment Cell Research held on 1-2 February at New Delhi.

Association of catalase T/C exon 9 and glutathione peroxidase codon 200 polymorphisms in relation to their activities and oxidative stress with vitiligo susceptibility in Gujarat population

Shajil EM[1], Naresh C. Laddha[1], Sreejatha Chatterjee[1], Amina R. Gani[1], Reem A. Malek[1], Bela J. Shah[2] and Rasheedunnisa Begum[1]*

[1]Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara, Gujarat 390002, India
[2]Department of Dermatology, STD and leprosy, BJ Medical College and Civil Hospital, Ahmedabad, Gujarat 300006, India
*Address correspondence to Rasheedunnisa Begum, e-mail: rasheeda@icenet.co.in

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Dear Sir,

Vitiligo is a common depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1–2% of the world population. The incidence of vitiligo is found to be 0.5–2.5% in India. Gujarat and Rajasthan states have the highest prevalence i.e. ~8.8% (Shajil et al., 2006a,c). Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction. We have earlier reported an alteration in the systemic antioxidant system with a significant reduction in catalase (CAT) and glutathione peroxidase (GPX) activities in vitiligo patients (Agrawal et al., 2004; Shajil and Begum, 2006). Here, we report a case-control study for the well-documented *CAT* exon 9 T/C (rs769217) and *GPX*1 codon 200 proline to leucine (rs1050450) polymorphisms and their relation to activities and lipid peroxidation (LPO) levels in Gujarat vitiligo patients where the prevalence of vitiligo is very high.

DNA was isolated from blood of 126 vitiligo patients and 143 age-matched healthy controls. Oligonucleotide primers used for PCR amplification of *CAT* exon 9 were 5′-GCCGCCTTTTTGCCTATCCT-3′ (forward primer) and 5′-TCCCGCCCATCTGCTCCAC-3′ (reverse primer) and for *GPXI* exon 2 were 5′-TGTGCCCCTACGCAGGTACA-3′ (forward primer) and 5′-CCAAATGACAATGACACAGG-3′ (reverse primer). For RFLP analysis, *CAT* exon 9 amplicon was digested with *Bst*XI and subjected to 15% PAGE; *GPXI* exon 2 amplicon was digested with *Apa*I and subjected to 2% AGE. Erythrocyte LPO, CAT, and GPX activities in vitiligo patients and controls were assayed by the standard methods (Agrawal et al., 2004).

The results of RFLP analysis of T/C silent substitution in *CAT* exon 9 (Asp389) is shown in Table 1. The observed allele frequencies of C and T were 0.867 and 0.133, respectively, in controls; 0.822 and 0.178 in vitiligo patients (Table 1). The allele frequencies of this T/C SNP did not differ significantly between the control and patient population ($p < 0.434$). When the observed control and patient genotype frequencies were compared with the expected values using a 3×2 contingency table in a standard chi-squared test (Table 1) they did not show any significant change ($p = 0.269$) suggesting that there is no possible association of the T/C exon 9 (*Bst*XI) *CAT* marker with vitiligo. The observed genotype frequencies of the control population did not differ significantly from those predicted by Hardy–Weinberg equation ($p < 0.259$) Also the patient population did not deviate from Hardy–Weinberg equilibrium ($p < 0.194$). The results of RFLP analysis of C/T polymorphism in *GPX* 1 exon 2 (Pro → Leu) is shown in Table 1. The observed *GPX* 1 allele frequencies of C and T were 0.721 and 0.279, respectively, in controls; 0.742 and 0.258 in vitiligo patients (Table 1). The allele frequencies of this T/C SNP did not differ significantly between the control and patient population ($p < 0.313$). When the observed control and patient genotype frequencies were compared with the expected values using a 3×2 contingency table in a standard chi-squared test (Table 1) they did not show any significant change ($p = 0.599$) suggesting that there is no association of the C/T exon 2 (*Apa*1) *GPX* 1 marker with vitiligo. The observed genotype frequencies of the control population differed significantly from those predicted by Hardy–Weinberg equation ($p < 0.0350$). Also the patient population is deviated from Hardy–Weinberg equilibrium ($p < 0.0037$).

Catalase activity did not show any significant change in vitiligo patients compared with controls. Also, CAT activity did not differ significantly between patients with C/C and C/T genotypes. However, GPX activity was found to be significantly decreased ($p < 0.0001$) in vitiligo patients compared with controls. Also, GPX activity was significantly decreased ($p < 0.0486$) in patients with C/C genotype compared with C/T genotype. LPO levels

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Table 1. Distribution of genotypes and alleles for the T/C SNP in *CAT* exon 9 and *GPX 1* exon 2 in vitiligo patients and control population

		Observed genotype counts			Observed allele frequencies		Expected genotype counts			
	N	C/C	C/T	T/T	C	T	C/C	C/T	T/T	p-value
Catalase										
Controls	143	106	36	1	0.867	0.133	107.54	32.89	2.57	0.259
Patients	126	82	43	1	0.822	0.178	85.18	36.92	3.91	0.194
p-value	0.269									
Glutathione peroxidase										
Controls	143	68	70	5	0.721	0.279	74.36	57.49	11.15	0.0350
Patients	126	63	61	2	0.742	0.258	69.3	48.26	8.44	0.0037
p-value	0.599									

showed significant increase ($p < 0.0008$) in vitiligo patients compared with controls, which is an index for oxidative stress. However, LPO levels did not differ significantly between patients with *GPX* 1 and *CAT* C/C and C/T genotypes.

Casp et al. (2002) and Gavalas et al. (2006) showed an association between vitiligo susceptibility and *CAT* T/C SNP at codon 389 in Caucasian population and English population respectively. However, in Korean population the T/C *CAT* exon 9 genotype distribution and allele frequencies were not significantly associated with vitiligo (Park et al., 2006), which is in accordance with our study. Thus it seems that the genotype distribution of *CAT* T/C is different in different ethnic groups. Our results vary from the Caucasian and English population may be because of the racial differences. This study also shows that there is no change in the CAT activity in vitiligo patients compared with controls and our results are in accordance with our previous studies (Agrawal et al., 2004; Amina et al., 2006). However, there are reports showing significant reduction in CAT activity in vitiligo patients compared with controls (Passi et al., 1998). Also patients with CC and CT genotypes have not shown any significant change in the CAT activity. The role of *GPX* 1 polymorphism is not addressed so far in vitiligo but based on our previous results (Agrawal et al., 2004) we have made an attempt to study its association with vitiligo. Nevertheless, Ratnasinghe et al. (2002) showed that the *GPX* 1 codon 200 polymorphism was associated with increased risk of lung cancer in Finnish population. It was also reported that leucine containing *GPX* 1 allele was more frequently associated with breast cancer than the proline containing allele (Hu and Diamond, 2003). Conversely, our study could not find any association of this polymorphism with vitiligo, however the proline containing *GPX*1 allele (C/C) showed significant reduction ($p < 0.0486$) in GPX activity in vitiligo patients compared with patients with C/T genotype. Surprisingly, this change in activity was not observed in C/C and C/T genotypes of control population. In addition, allelic variants are reported in the *GPX 1* gene with low levels of GPX activity (Hu and Diamond, 2003; Ratnasinghe et al., 2002). Thus our results suggest that other SNPs in vitiligo patients could be contributing for the reduced GPX activity. Our results show a significant increase in LPO levels in vitiligo patients compared with controls. In patients *CAT* C/C genotype showed higher LPO levels compared with C/T genotype. However, no change is observed in LPO levels in vitiligo patients with different genotypes of *GPX* 1.

The present study shows that the well-documented *CAT* exon 9 T/C and *GPX* 1 codon 200 polymorphisms may not be associated with Gujarat vitiligo patients where the prevalence of vitiligo is alarmingly high. These results suggest for the presence of novel SNPs in Gujarat vitiligo population. Ours is the first report on *CAT* and *GPX* 1 polymorphisms in Gujarat vitiligo patients.

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ACETYLCHOLINE ESTERASE LEVELS IN DIFFERENT CLINICAL TYPES OF VITILIGO IN BARODA, GUJARAT

E M Shajil, Y S Marfatia*, Rasheedunnisa Begum

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Abstract

Vitiligo is an acquired depigmentary disorder involving a progressive loss of melanocytes from the epidermis and hair follicles. Gujarat has a high prevalence of vitiligo. One of the major hypotheses in the pathogenesis of vitiligo is the neurochemical hypothesis. According to the neural hypothesis neurochemical mediator/s such as acetylcholine secreted by the nerve endings cause the destruction of melanocytes. Acetylcholine esterase (AChE) activity has been found to be lowered in vitiligo patients during the process of depigmentation. We have earlier reported impairment of systemic antioxidant status in Baroda vitiligo patients, and we now show analysis of blood AChE activity in these patients. The study consisted of 121 vitiligo patients and 126 age and sex-matched healthy controls. Acetylcholine esterase activity showed significant decrease in vitiligo patients. However, there is no significant difference in AChE activity in segmental and non-segmental types as well as in active and stable types of vitiligo. The age group 16-25 showed a significant decrease in AChE activity. This study suggests that AChE may be inactivated due to high systemic oxidative stress in these patients. This is the first report showing that AChE may be playing a role in the pathogenesis of vitiligo in Baroda patients.

Key Words: *Acetylcholine esterase, clinical-types, neural hypothesis, vitiligo*

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Introduction

Vitiligo is a depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1-2% of the world population.[1] The incidence of vitiligo is found to be 0.5-2.5% in India.[2] Gujarat and Rajasthan states have the highest prevalence ~8.8%.[3] Vitiligo is classified into non-segmental and segmental clinical types.[4] Non-segmental type includes vulgaris, acrofacial, focal, and universal sub-types. In vulgaris the lesions are found in typical zones with symmetrical distribution. Acrofacial sub-type of non-segmental vitiligo affects face and distal extremities. In focal vitiligo one or more patches are found in one area but not in segmental pattern. In universal vitiligo the depigmentation involves more than 80% of the body.[5] In segmental vitiligo one or more macules are found in dermatomal unilateral distribution.

*From the Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara, *Department of Skin and VD, Sir Sayajirao Gaikwad Medical College, Vadodara, Gujarat, India.* ***Address correspondence to:*** *Dr. Rasheedunnisa Begum, Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. E-mail: rasheeda@icenet.co.in*

Though vitiligo has been extensively studied in the past five decades, its etiology is still being debated.[6-8] Several hypotheses are proposed about the pathogenesis of vitiligo and oxidative stress hypothesis considers a systemic involvement during the course of the disease.[9-11] According to Taieb[6] the origin of segmental and non-segmental vitiligo may be different. Local/systemic factors affect the homeostasis of the epidermal melanin unit in segmental vitiligo whereas an impaired redox status of the epidermal melanin unit acts as the primary defect further leading to inappropriate immune response in non-segmental vitiligo. The neural theory is more related to segmental vitiligo whereas the autoimmune theory is involved in non-segmental vitiligo.[6] There are no reports on systemic acetylcholine esterase (AChE) levels in vitiligo patients. Hence, we have made an attempt to explore whether there is any involvement of systemic AChE in precipitating vitiligo. In this study we show analysis of blood AChE activity in different age groups of vitiligo patients compared to controls; in segmental and non-segmental types; and in active and stable forms of vitiligo.

Materials and Methods

For the estimation of blood AChE activity, 121 vitiligo

patients were divided into different age groups (5-15, 16-25, 26-35, 36-45, and 46-55 years) and clinical types after written informed consent has been obtained. The patients had no other associated diseases. The study included 126 age and sex-matched healthy consenting volunteers as controls. Blood AChE activity was assayed according to the method of Reiner *et al.*[12] Briefly, AChE catalyses the hydrolysis of acetylcholine to thiocholine and thiocholine in turn reacts with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to produce yellow color due to the formation of 5-thio-2-nitrobenzoic acid. Rate of formation of the yellow anion is measured at 412 nm and the activity of AChE is expressed as micromoles of acetylthiocholine hydrolyzed (ATCh) per gram hemoglobin per minute.

Statistical analysis

Results in vitiligo patients and controls were compared using the paired student's *t*-test. One way analysis of variance (ANOVA) was used to determine significant differences in AChE enzyme activities between different age groups and different clinical types of vitiligo utilizing statistical software program, Prism and $P \leq 0.05$ was considered significant.

Results

The present study is an attempt to analyze blood AChE activity in vitiligo patients compared to controls. Acetylcholine esterase showed significant decrease in vitiligo patients compared to controls (Table 1). Patients were also classified into different clinical types for our further analysis. However, there is no significant difference in the AChE activity among different clinical types (Table 1), between segmental and non-segmental types (Table 1) as well as between active and stable vitiligo (Table 1). We have also analyzed the role of AChE in genetic predisposition of vitiligo, by comparing its activity in patients with positive family history with those with negative family history. However no significant difference was observed in AChE activity of these two groups of patients (Table 1). Further, AChE activity in different age groups of vitiligo patients has been analyzed (Table 2) and the age group 16-25 showed a significant decrease in AChE activity compared to controls. Other age groups have not shown any difference in the activity compared to controls (Table 2). Our analysis by ANOVA in different age groups and different clinical types showed no significant difference in the AChE activity among the age groups and clinical types.

Table 1: Blood acetylcholine esterase activity in different clinical types, active and stable types of vitiligo patients compared to controls

Clinical types of vitiligo	Blood AChE activity
Control	41.69 ± 0.778 (n=126)
Patients (Total)	39.48 ± 0.624 (n=121)* P<0.0281
Segmental type	38.82 ± 2.588 (n=10)
Non-segmental type (NS)	40.12 ± 0.709 (n=111)
NS- universal	38.31 ± 7.430 (n=3)
NS- focal	39.89 ± 1.863 (n=19)
NS- vulgaris	40.26 ± 0.866 (n=71)
NS- Acrofacial	40.15 ± 1.704 (n=18)
Active	39.26 ± 0.912 (n=60)
Stable	39.69 ± 0.859 (n=61)
Patients with positive family history	41.19 ± 1.210 (n=29)
Patients with negative family history	38.94 ± 0.721 (n=92)

Values are expressed as mean ±SE, acetylcholine esterase activity is expressed as micromoles of acetylthiocholine hydrolyzed per gram hemoglobin per minute

Discussion

Melanocytes are neural crest derived cells with an embryological link to the nervous system.[13] According to neural hypothesis, neurochemical mediators including acetylcholine secreted by the nerve endings are toxic to melanocytes leading to their destruction in vitiligo patients. Thus segmental vitiligo may be associated with the dysfunction of cholinergic sympathetic nerves.[6,14] Acetylcholine esterase activity was found to be lowered in vitiliginous skin during depigmentation,[15] suggesting that acetylcholine may aggravate the process of depigmentation in vitiligo.[15] Further, a possible cholinergic involvement in vitiligo has been reinforced by Elwary *et al.* by demonstrating decreased sweating in the depigmented epidermis of these patients.[16] Schallreuter *et al.* studied H_2O_2 regulation of AChE and showed H_2O_2 mediated oxidation of AChE, thus emphasizing the role of oxidative stress in precipitating vitiligo.[17] It was proposed that inactivation of AChE is due to the oxidation of Trp^{432}, Trp^{435} and Met^{436} residues of the enzyme by H_2O_2.[17] It has also been shown that acetylcholine has an inhibitory effect on Dopa oxidase activity in the melanocytes and inhibits the pigment production.[15]

Our present study shows a significant decrease in the

Table 2: Blood acetylcholine esterase activity in different age groups of vitiligo patients compared to controls

Age groups	Controls	Vitiligo patients
5-15	41.88 ± 1.898 (n=19)	38.64 ± 0.9040 (n=22)
16-25	43.94 ± 1.614 (n=45)	38.48 ± 0.9921 (n=55)** P<0.0035
26-35	42.31 ± 1.694 (n=24)	40.76 ± 1.813 (n=18)
36-45	40.74 ± 1.472 (n=25)	41.98 ± 2.212 (n=15)
46-55	37.32 ± 1.577 (n=13)	40.30 ± 1.206 (n=11)

Values are expressed as mean ±SE, acetylcholine esterase activity is expressed as micromoles of acetylthiocholine hydrolyzed per gram hemoglobin per minute

blood AChE activity in vitiligo patients compared to controls (Table 1), nevertheless our earlier reports[9,18,19] showed significant increase in the lipid peroxidation levels[9,18] in the same vitiligo patients which was an indicator of oxidative stress. This report in conjunction with our earlier studies[9,18,19] provides evidence that AChE may be inactivated due to high systemic oxidative stress in these patients. Acetylcholine thus accumulated may lead to the destruction of melanocytes resulting in the precipitation of vitiligo in these patients. However we could not find any significant change in the AChE activity in segmental vitiligo (Table 1), which is attributed to be of neurochemical origin.[6] This is the first report showing that systemic AChE may be playing a role in the pathogenesis of Baroda vitiliginous patients.

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Original Article

VITILIGO: CLINICAL PROFILES IN VADODARA, GUJARAT

E M Shajil, Deepali Agrawal, Krishna Vagadia*, Y S Marfatia*, Rasheedunnisa Begum

Abstract

Purpose: Vitiligo is an acquired depigmentary condition involving a progressive loss of melanocytes from the epidermis and hair follicles We have earlier reported impairment of systemic antioxidant status of Baroda vitiligo patients (*Pigment Cell Res 2004; 17; 289-94)* and we now show analysis of the clinical profiles of these patients. **Procedure:** The study comprised of 424 vitiligo patients. Clinical and demographic details of all the patients were obtained from the vitiligo clinical proformas. Lipid peroxidation levels (LPO) in erythrocytes of vitiligo patients and healthy controls were estimated. **Result:** Out of four hundred and twenty four outpatients, males constituted 38.44% and females were 61.56%. Mean age of the patients was 25.59 years. The sites of onset were the lower limb, face, trunk, upper limb, genital, hand, labia and scalp in the descending order of frequency. Koebner's phenomenon was observed in 12.74%, diabetes mellitus in 1.18%, leukotrichia in 9.2% and premature graying of hair in 23.11% patients. A family history of vitiligo was present in 21.93% of the patients. Significant increase ($P<0.002$) in the LPO levels of the vitiliginous patients was observed compared to the controls. **Conclusion:** Vitiligo vulgaris was the most common form of the disease which constituted 52.36% of the patients followed by focal vitiligo (28.54%), segmental vitiligo (6.84), acrofacial (7.55%), mucosal (2.83%) and universal vitiligo (1.89%). Systemic oxidative stress may have a pathophysiological role in precipitating all clinical types of vitiligo in Vadodara vitiliginous patients.

Key Words: *Vitiligo, Clinical types, Precipitating factors, Oxidative stress*

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Introduction

Vitiligo is an idiopathic, acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches of different sizes and shapes and affects 1-2% of the world population.[1,2] Based on a few dermatological outpatient records, the incidence of vitiligo is found to be 0.25-2.5% in India.[3,4] Gujarat and Rajasthan states have the highest prevalence ~ 8.8%.[5] There is a stigma attached to vitiligo and affected persons and family, particularly girls are socially ostracized for marital purpose.[6] Since Gujarat shows high prevalence of vitiligo in India, we have undertaken a systematic study to understand the etiopathogenesis of vitiligo. In this context we have reported the age-dependent antioxidant parameters of vitiligo patients in Vadodara, and found impairment in the systemic antioxidant status of the patients suggesting that melanocyte damage in vitiligo may be linked with generalized oxidative stress.[7-9]

The present report is an effort to learn more about its precipitating factors and clinical features of Vadodara vitiligo patients.

*Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, *Department of Skin and VD, Sir Sayajirao Gaikwad Medical College, Vadodara, Gujarat - 390002, India.* ***Address correspondence to****: Rasheedunnisa Begum, India. E-mail: rasheeda@wilnetonline.net.*

Materials and Methods

The study consisted of 424 vitiligo patients who visited the Sir Sayajirao Gaikwad Medical College hospital Skin and Venereal OPD and a service hospital in Vadodara. The diagnosis was essentially clinical and demographic details of all the patients including the age of onset, initial site of onset, duration of the disease, precipitating factors, and presence of leukotrichia, Koebner's phenomenon, halo nevi, other associated diseases and family history were obtained from the vitiligo clinical proformas. Lesions confined to one or a few patches localized in a particular area were grouped as focal vitiligo; lesions distributed in a segmental/dermatomal pattern as segmental vitiligo; lesions noted over both face and acral regions as acrofacial vitiligo; lesions affecting many parts of the body as vitiligo vulgaris and lesions confined only to mucous membranes as mucosal vitiligo.[4]

Blood samples were collected from one hundred and twenty three vitiligo patients without any other associated disaeses and also from one hundred and six age matched healthy consenting volunteers for the estimation of Lipid peroxidation (LPO) levels. Written consent had been obtained from the vitiligo patients. LPO levels in erythrocytes were estimated according to the procedure of Beuge and Aust.[10] Malonaldehyde produced during peroxidation of lipids served as an index of LPO. Methylenedioxyamphetamine (MDA) reacts with

hiobarbituric acid to generate a colored product, which vas measured in 532 nm and the results were expressed as moles MDA formed/g Hb/ min.

Results

Among the patients 61.56% (261) were women and 38.44% (163) were men. The female to male ratio was 1.6:1. Duration of the disease varied from 15 days to 60 years and the average disease duration at the time of hospital visit was 3.3 years. Age and sex distribution of the patients along with various clinical types of vitiligo is shown in Table 1. One hundred and fifty two women (58.23%) reported the disease in the second (35.63%) and third (22.60%) decades as compared to 29.45 and 22.70% of men respectively. Out of 163 men 85 (52.15%) were in their second and third decades. The mean age of the disease was 25.59 years.

A detailed clinico-demographic profile of the patients is given in Table 2. Vitiligo vulgaris was the most common form of the disease in 222 (52.36%) patients. The sites of onset were the lower limb (45.52%), face (20.04%), trunk (12.03%), upper limb (8.96%), genital (5.19%), hand (3.54%), labial (2.83%) and scalp (1.87%) in the descending order.

Around 61 (21.93%) patients had a positive family history of vitiligo. The first-degree relatives (parent/brother/sister/son/daughter) were affected in 58 (13.68%), second-degree relatives (grandparent/maternal and/or paternal uncle or aunt) in 24 (5.66%) and third degree relatives in 11 (2.59%) patients, (Table 3).

Leukotrichia was seen in 39 (9.20%) patients, of these 23 (5.42%) were males and 17 (4.00%) females. Koebner phenomenon was observed in 54 (12.75%) patients, out of which 31 (7.31%) were males and 23 (5.42%) females. Premature graying of hair was observed in 98 (23.11%) patients. Of the various diseases associated with vitiligo, diabetes mellitus was found in 5 (1.18%), thyroidism in 4 (0.94%), tuberculosis in 4 (0.94%) hypertension in 3 (0.71%), psoriasis in 1 (0.24%), bronchial asthma in 3 (0.71%) and halo nevus in 2 (0.47%) patients (Table 4). Major precipitating factor was found to be physical injury (15.33%), others included emotional tension, plastic footwear, etc. (Table 5). Association of Koebner phenomenon and leukotrichia was observed in 12.74 and 9.2% of the patients respectively, (Table 6). A significant increase in lipid peroxidation levels was observed in vitiligo patients compared to controls ($P<0.002$) and also in different clinical types of vitiligo i.e., vulgaris ($P<0.002$), focal ($P<0.01$), segmental ($P<0.03$), acrofacial, ($P<0.01$) and universal ($P<0.03$) vitiligo (Table 7).

Discussion

The prevalence of vitiligo is high in India. The relative prevalence varies between 0.46 to 8.8%.[4] The varying ethnic backgrounds of the population residing in different geographic regions with varying environmental conditions may contribute to the wide variation in the prevalence of vitiligo in India.

The female to male ratio in our study is 1.6:1, which is different from that reported by Handa and Kaur,[4] Koranne *et al*[11] and Sarin *et al.*[12] Thus, most of the reports showed that males and females were affected with almost equal frequency.[3,6,13-16] The number of female vitiligo patients reported at the hospitals in Baroda was found to be higher than males and females predominate presumably because women notice the change in appearance and approach the doctors sooner than men. In 55.90% of the patients, the age of onset was in the second or third decade, consistent with the most reports from India[11,12,17] and the west.[13,16,18] This shows that the disease starts at a younger age in the Indian population. However, Howtiz *et al*[15] showed the age of onset to be between 40-60 years.

The duration of the disease varied widely from 2 weeks to

Table 1. Age and sex distribution of patients with various clinical types of vitiligo

Age group	Clinical types of vitiligo											
	Vulgaris		Focal		Segmental		Mucosal		Acro facial		Uni versal	
	M	F	M	F	M	F	M	F	M	F	M	F
0-10	12	14	6	13	0	3	-	1	1	0	0	0
12-10	17	50	17	30	7	7	3	-	4	5	0	0
21-30	23	33	9	12	0	4	-	3	5	4	0	3
31-40	17	21	3	13	1	2	1	3	1	5	0	3
41-50	15	11	5	8	3	1	-	-	1	4	1	0
51-60	4	4	1	1	1	0	-	-	1	0	1	0
51-70	0	1	2	1	0	0	-	-	1	0	0	0
>70	0	0	0	0	0	0	-	-	0	0	0	0
Total	88	134	43	78	12	17	4	8	14	18	2	6

Table 2. Clinico demographic profile of vitiligo patients

	Clinical types of vitiligo					
No. of patients n=424	**Vulgaris (%)**	**Focal (%)**	**Segmental (%)**	**Mucosal (%)**	**Acro facial (%)**	**Universal (%)**
	222	121	29	12	32	8
	(52.36)	(28.54)	(6.84)	(2.83)	(7.55)	(1.89)
Males n=163	88	43	12	4	14	2
	(20.75)	(10.14)	(2.83)	(0.94)	(3.30)	(0.47)
Females n=261	134	78	17	8	18	6
	(31.60)	(18.40)	(4.00)	(1.89)	(4.24)	(1.41)
Site of onset						
Scalp	3	4	1	0	0	0
	(0.71)	(0.94)	(0.24)			
Face	34	29	8	0	14	0
	(8.02)	(6.84)	(1.89)		(0.11)	
Hand	7	2	0	0	6	0
	(1.65)	(0.47)			(1.42)	
Trunk	25	18	6	0	0	2
	(5.90)	(4.24)	1.41)			(0.47)
Upper limb	14	18	1	0	4	1
	(3.30)	(4.24)	(0.24)		(0.94)	(0.24)
Lower limb	123	45	9	32	8	5
	(29.00)	(10.61)	(2.12)	(0.71)	(1.89)	(1.18)
Mucosal labial						
	4	3	3	2	0	0
	(0.94)	(0.71)	(0.71)	(0.47)		
Genital	12	2	1	7	0	0
	(2.83)	(0.47)	(0.24)	(1.65)		
Total	222	121	29	12	32	8

60 years, with the mean duration of 3.3 years in this study. The slow progression in the rate of disease may be the underlying reason for showing long duration of vitiligo in this study.

In our study vitiligo vulgaris was the most common type observed followed by focal, acrofacial, segmental, mucosal and universal. The frequency of distribution of clinical types of vitiligo varies in different studies.[11,12,18,19] According to the reports of Koranne *et al*[11] and Sarin *et al*[12] generalized vitiligo was found to be more common. Thus our results suggest that Indians not only have an increased incidence of the disease but also have more widespread disease.

Lower limbs were the most common site of onset in 45.52% of the out patients in this study irrespective of the clinical type of vitiligo. This is in concordance with the studies by Behl and Bhatia,[17] Dutta and Mandal[14] and Lerner,[20] however, it is at variance with Handa and Kaur[4] and Sehgal,[19] in which the face was found to be most common site of onset. In Western studies also extremities were the most commonly involved sites of onset.[18,20]

Table 3. Family history of vitiligo patients

First degree relatives	N (%)	%
Parents	40 (9.43)	
Brother	6 (1.42)	13.68
Sister	7 (1.65)	
Children	5 (1.17)	
Second degree		
Paternal grandparent	9 (2.12)	
Maternal grandparent	8 (1.89)	5.66
Maternal/paternal uncle/aunt	7 (1.65)	
Third degree		
Cousin	9 (2.12)	
Niece	0 (0)	2.59
Nephew	2 (0.47)	
Total	93	21.93

Table 4. Association of vitiligo with other diseases

Associated diseases	N	%
Diabetes mellitus	5	1.18
Thyroidism	4	0.94
Tuberculosis	4	0.94
Hypertension	3	0.70
Alopecia areata	5	1.18
Diabetes with hypertension	1	0.24
Psoriasis vulgaris	1	0.24
Bronchial asthma	1	0.24
Halo nevus	2	0.47

Table 5. Precipitating factors

Precipitating factors	N	%
Physical trauma	65	15.33
Plastic shoes	8	1.89
Pressure	10	2.36
Emotional/mental trauma	3	0.70
Drug/chemical	2	0.47
Bindi	2	0.47
Burn	1	0.24
Condom	2	0.47
Pregnancy	1	0.24

Table 6. Association of koebner phenomenon and leukotrichia with vitiligo

Association with	N	%
Koebner phenomenon	54	12.74
Leukotrichia	39	9.20

Table 7. Lipid peroxidation levels in various clinical types of vitiligo patients

Control	187.7 ± 3.00
N = 106	
Test	204.2 ± 6.40
n=123	$P<0.002$
Vulgaris	201.8 ± 4.40
n=72	$P<0.002$
Focal	204.3 ± 7.48
n=19	$P<0.01$
Segmental	206.1 ± 9.00
n=10	$P<0.03$
Acrofacial	204.6 ± 8.71
n=18	$P<0.01$
Universal	218.6 ± 6.47
n=4	$P<0.03$

LPO levels are expressed as nmoles of malonaldehyde formed/g Hb/min

Leucotrichia was seen in 9.2% of the patients in this study. Leucotrichia was reported in 9-45% of patients with vitiligo.[14,19] Leucotrichia is considered to be a poor prognostic factor. Koebner phenomenon was seen in 12.74% of Baroda patients. Koebner phenomenon was reported to occur as many as 33% of vitiligo patients[13,16] which is a common feature found in active vitiligo.

The association of vitiligo with thyroid disease was 0.94% in our study, which was reported to be 7.8% by Schallreuter *et al.*[21] Diabetes mellitus was found to be 1.18% in our study, whereas the reported value was 0.6%.[21] Premature graying and hypertension were found to be 23.11 and 0.70% in our study, but premature graying was reported to be 2.8%.[21] Tuberculosis and psoriasis were found to be 0.94, 0.24%, and the incidence of bronchial asthma was found to be 0.71% in our study. We observed halo nevi in 0.47% of our patients. Halo nevi in vitiligo have been reported to occur commonly and to be single or multiple.[11,16,17]

There was a family history of vitiligo in 21.93% of Vadodara patients and 20 patients had more than one family member with the disease. Vitiligo has a polygenic or autosomal dominant inheritance pattern with incomplete penetrance and variable expression.[13,16,18] Familial occurrence has been reported to vary from 6.25-30%.[11,12,14,17,19,20,22] Positive family history is considered to be a poor prognostic factor.

A significant increase in lipid peroxidation levels was observed in vitiligo patients compared to controls ($P<0.002$) (Table 7). However, non significant changes in lipid peroxidation levels were reported by Picardo *et al.*[23] Systemic oxidative stress may have a pathophysiological role in precipitating all clinical types of vitiligo in Vadodara vitiliginous patients.

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Review Article

Vitiligo: Pathomechanisms and genetic polymorphism of susceptible genes

E M Shajil[1], Sreejata Chatterjee[1], Deepali Agrawal[1], T Bagchi[2] & Rasheedunnisa Begum[1*]

[1]Department of Biochemistry, [2]Department of Microbiology and Biotechnology Center, Maharaja Sayajirao University of Baroda, Vadodara 390 002, India

Vitiligo is a depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1-4% of the world population. Incidence of vitiligo is found to be 0.5-2.5% in India with a high prevalence of 8.8% in Gujarat and Rajasthan states. The cellular and molecular mechanisms that lead to melanocyte destruction in this disorder are not yet been fully elucidated. Genetic factors, neural factors, toxic ROS metabolites, autoantibodies and autoreactive T lymphocytes may be the causative agents for the selective destruction of melanocytes. Three major hypotheses of pathogenesis of vitiligo are neural, autoimmune and oxidative stress hypotheses, however none of them explains the pathogenesis of vitiligo *in toto*. Genetics of vitiligo is characterized by incomplete penetrance, multiple susceptibility loci and genetic heterogeneity. Recent advances in this field are linkage and association of candidate gene studies. The linkage and association studies provide a strong evidence for the presence of multiple vitiligo susceptibility genes on different chromosomes. Several candidate genes for vitiligo are identified from different populations. In this review, we have provide an overview of different hypotheses of vitiligo pathogenesis, and discuss the recent advances in this field with special reference to linkage, association and candidate gene approach.

Keywords: Autoimmunity, Neural factors, Oxidative stress, Polymorphism, Vitiligo

Introduction

Vitiligo is an acquired, idiopathic, hypomelanotic disease characterized by circumscribed depigmented macules[1]. Absence of melanocytes from the lesional skin due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo[2]. The lesions may be progressive and may develop at any age, however in many cases the onset is reported at the second decade of the life[3]. In India there is a stigma associated with vitiligo and affected persons and their families particularly girls are socially ostracized for marital purpose[4]. The etiology of vitiligo is still unknown, but genetic factors, oxidative stress, autoimmunity, neurological factors, toxic metabolites and lack of melanocyte growth factors might contribute for precipitating the disease in susceptible people[5].

Prevalence

Vitiligo affects approximately 1-4% of the world population[1]. Its prevalence is varying from 0.46 to 8.8% in India[6]. The Gujarat and Rajasthan states have the highest prevalence i.e. around 8.8%[7].

Types of vitiligo

Vitiligo is classified according to the distribution, pattern and extent of depigmentation. There are many reports on classification. However, most investigators distinguished two large subtypes of vitiligo; segmental vitiligo (SV) and non-segmental vitiligo (NSV)[8] as shown in Table 1. According to another classification proposed by Nordlund and Lerner[9] three types are identified i.e., localized, generalized and universal vitiligo (Table 2). Localized vitiligo is further classified as shown in Table 2 into focal and segmental; generalized into acrofacial, vulgaris and mixed subtypes.

Genetics of vitiligo

Prevalence of familial cases of vitiligo varies from 6.25-38% (ref. 1). About 20% of patients have at least one first-degree relative with vitiligo. The relative risk of vitiligo for the first-degree relatives of patients is

Table 1—Clinical subtypes of vitiligo

Segmental vitiligo	Non-segmental vitiligo
Begins often in the childhood	Later onset
Autoimmunity rare	Autoimmunity associated
Frequently facial	Trauma prone sites and koebnerisation
Stable results after autologus grafting	Unstable results after autologus grafting
Dermatomal, unilateral distribution	Non-dermatomal bilateral distribution

*Correspondent author: E-mail: rasheeda@icenet.co.in

Table 2—Clinical classification of vitiligo

Localized		Generalized			Universal
Focal	*Segmental*	*Acrofacial*	*Vulgaris*	*Mixed*	
One or more patches in one area but not in segmental pattern	One or more macules in dermatomal, unilateral distribution.	Affects face and distal extremities	Symmetrical distribution of lesions in typical zones	Segmental along with vulgaris or acrofacial	Involves more than 80% of the body

increased by at least 7-10 fold[10]. The inheritance pattern of vitiligo does not follow the simple Mendelian pattern and its mode of heredity suggests that it is a polygenic disease. Vitiligo seems to be a complex hereditary disease governed by a set of recessive alleles situated at several unlinked autosomal loci which may be involved in the generation of oxidative stress, melanin synthesis, autoimmunity etc. that could collectively confer the vitiligo phenotype[10].

Melanocytes

Melanocytes are the melanin producing cells. Apart from the skin, melanocytes are present in retinal pigment epithelium, uveal tract, inner ear and leptomeninges. In the skin they reside in the matrix of the hair follicle of the basal layer of epidermis. Melanocytes are highly dendritic and these dendrites project into the malphigian layer of epidermis where they transfer the melanosomes to keratinocytes[11]. Each epidermal melanocyte secretes melanosomes to approximately 36 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit.

Tyrosinase is the key enzyme required for melanin production. The different steps of melanin production are shown in Fig 1. Tyrosinase catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA), which is the rate-limiting step for the melanin synthesis[12]. DOPA undergoes oxidation to dopaquinone, which is immediately converted into dopachrome and then to 5, 6 dihydroxy indole (DHI). Also tyrosinase related protein 2 (TRP 2) converts dopachrome to dihydroxy indole carboxylic acid (DHICA). DHI and DHICA further polymerize to form eumelanin. The switch between eumelanogenesis and pheomelanogenesis occurs at dopaquinone stage. Cysteine/glutathione reacts with the dopaquinone to produce cysteinyldopas that may undergo further cyclisation to benzothiazines and higher condensates giving rise to pheomelanins[12]. The major function of melanin is to confer photo protection to the skin from ionizing radiations[12].

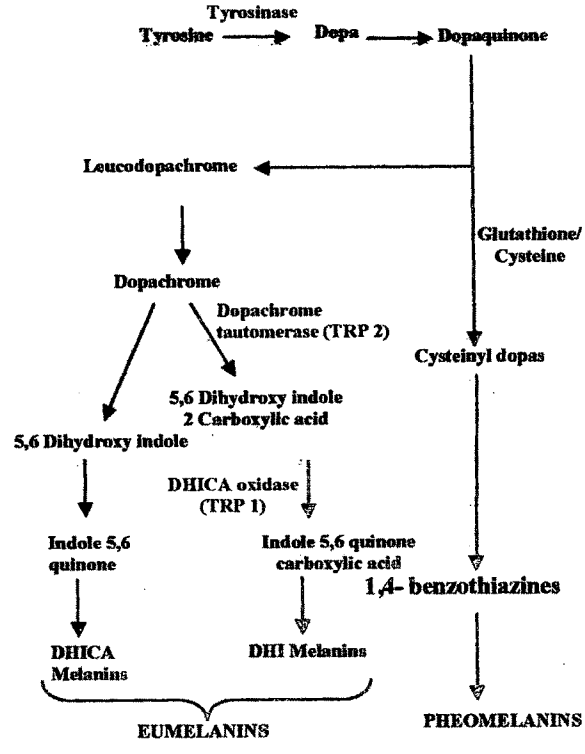

Fig. 1—Schematic representation of the melanogenic pathway showing major intermediates and enzymes involved in the process. The enzymes involved in the biogenesis of melanin are tyrosinase and tyrosinase related proteins TRP 1 and TRP 2.

Etiopathogenesis of vitiligo

Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated. The three main prevailing theories of pathogenesis of vitiligo are centered on neurochemical, autoimmune and oxidative stress aspects, but none of these hypotheses explain the entire spectrum of the vitiligo disorder.

Neurochemical hypothesis—Neurochemical mediators that are secreted by the nerve endings such as norepinephrine and acetylcholine are toxic to melanocytes (Fig. 2). Recent studies on vitiligo patients showed higher levels of plasma and urinary catecholamines and their metabolites especially at the

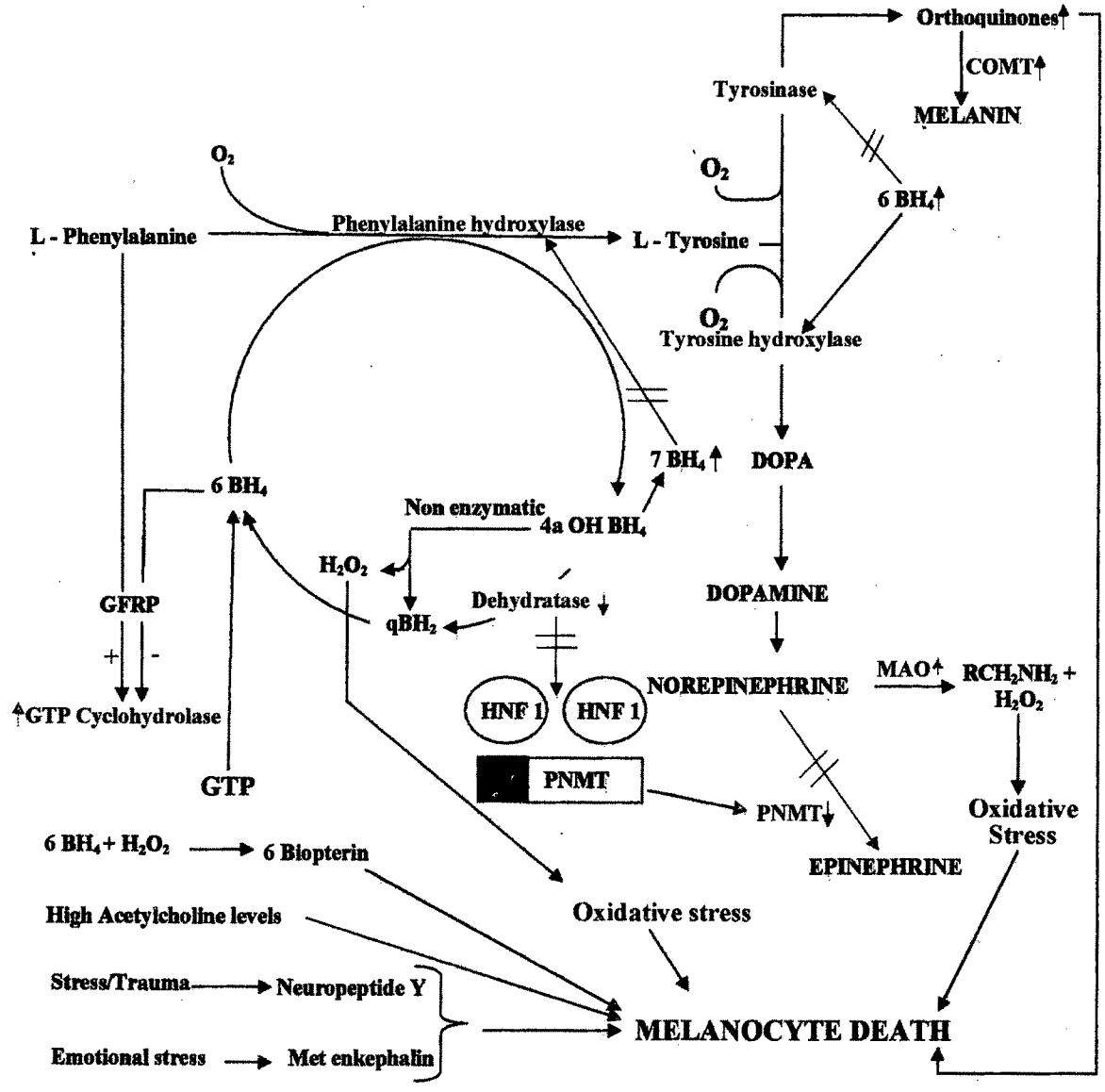

Fig. 2—The mechanism of the accumulation of norepinephrine, defective recycling of 6BH4, concomitant release of H_2O_2 and other factors which contribute to the neurochemical hypothesis of vitiligo. 6 BH4 is the cofactor for both phenylalanine hydroxylase (PAH) and tyrosine hydroxylase (TH). The *de novo* synthesis of 6 BH4 is controlled by GTP cyclohydrolase through the feedback regulation by growth factor regulatory protein (GFRP). Once formed, 6BH4 is recycled via the rate limiting enzyme 4a hydroxy 6 BH4 dehydratase (DH). DH levels are found to be lower in vitiligo. During this metabolic step 7 BH4 is formed non-enzymatically from the intermediate 4a carbinolamine which is dehydrated by DH to quinonoid dihydropterin (qBH2). 7 BH4 can inhibit PAH. Consequently the short circuit to qBH_2 causes the formation of H_2O_2 from O_2. 6BH4 will inturn reacts with H_2O_2 to form 6 biopterin which is toxic to melanocytes. DH also controls the transportation of the PNMT (Phenylethanolamine N methyl transferase) gene via the transcription factor hepatocyte nuclear factor 1 (HNF 1). In vitiligo DH is defective which results in the increased synthesis of 6BH4 leading to the stimulation of catecholamine pathway via the activation of tyrosine hydroxylase (TH). Tyrosinase is regulated by 6BH4 through uncompetitive inhibition whereas tyrosine hydroxylase requires 6BH4 as cofactor. The downregulation of PNMT due to defective trascription of the PNMT gene causes increased norepinephrine which in turn induces catecholamine degrading enzymes monoamine oxidase A (MAO A) and Catechol O methyltrasferase (COMT). MAO A oxidizes norepinephrnie which yields H_2O_2 that contributes to the melanocyte damage. Elevated COMT levels may be required for the detoxification of orthoquinones. And if the increased activity is insufficient, the presence of toxic orthoquinones can still contribute to the damage of melanocytes. The other factors contribute to the melanocyte death are high acetylcholine levels, met enkephalin and neuropeptide Y.

onset and in the active stage of the disease[13,14]. High concentrations of norepinephrine and its metabolites in vitiligo patients may be due to a reduction in phenylethanolamine-N-methyl transferase (PNMT) activity and an increase in tyrosine hydroxylase (TH) activity. These enzymes play a key role in production of L-dopa form, L-tyrosine[15]. Also (6R)-5,6,7,8-tetrahydrobiopterin (6BH4), the rate limiting cofactor/electron donor for TH is increased due to decreased 4a-hydroxy-6BH4 dehydratase (DH) activity in vitiligo patients[16]. There is also a defective recycling of 6BH4 which leads to increased non-enzymatic production of 7BH4, an isomer, concomitant with an increased production of H_2O_2 (Fig. 2). Presence of 7BH4 in the epidermis seems to initiate the process of depigmentation in vitiligo patients by blocking the supply of L-tyrosine to melanocytes. These alterations seem to cause melanocyte destruction in vitiligo[15]. Increased levels of norepinephrine also appear to induce another catecholamine degrading enzyme, monoamine oxidase (MAO)[17]. Keratinocytes and melanocytes in the depigmented skin exhibit increased monoamine oxidase-A activity which further causes keratinocytes to produce 4-fold more norepinephrine and 6.5-fold less epinephrine than control keratinocytes[18]. Norepinephrine is reported to be toxic to melanocytes. Increased MAO-A activity favours the formation of hydrogen peroxide, which too is toxic to melanocytes[18]. Moreover, the induced damage to melanocytes may not be buffered by low epidermal catalase levels[18]. A derangement of the enzyme dealing with catabolism of adrenergic transmitters namely catechol-*o*-methyl transferase (COMT) is also reported. COMT normally prevents the formation of toxic *ortho* quinones during melanin synthesis. Vitiligo patients show higher epidermal COMT activity, probably induced in the tissues by elevated levels of catecholamines secreted by keratinocytes or by nerve endings[19].

Aberrations in beta-endorphin and met-enkephalin secretion are also reported in vitiligo patients[20]. The met-enkephalin levels are found to be higher and this abnormality may be correlated with the emotional stress, which precipitates vitiligo in some patients. Abnormalities of neuropeptides are also observed in perilesional skin and blood of vitiligo patients[21]. The NPY released by either exogenous stimulus like trauma (e.g. Koebner phenomenon) or by endogenous stimulus (eg. stress)[21] alters the balance of neuropeptides in vitiliginous skin[22]. Neuropeptides are also reported to have immunoregulatory effects[23] (Fig. 2).

Oxidative stress hypothesis—Oxidative stress is considered to be the initial pathogenic event in the melanocyte destruction[15,24] as H_2O_2 accumulation is observed in the epidermis of active vitiligo patients[25]. Defective recycling of tetrahydrobiopterin in vitiligo epidermis is associated with the intracellular production of H_2O_2 (Fig. 2)[26]. In addition, an alteration in the antioxidant system, with a significant reduction in catalase activity has been demonstrated in both lesional and non-lesional epidermis of vitiligo patients[27] as well as in melanocytes derived from patients[24]. Antioxidant imbalance in peripheral blood mononuclear cells of active vitiligo patients is also observed. An increased intracellular production of reactive oxygen species appeared to be due to mitochondrial impairment[28]. These findings support the concept of a possible systemic oxidative stress in vitiligo. We reported systemic oxidative stress in vitiligo patients due to an imbalance in enzymatic and non-enzymatic antioxidant systems[29,30]. Our recent study suggests that the mechanism for generation of oxidative stress is different in different clinical types of vitiligo. The low levels of catalase may contribute for the generation of oxidative stress in segmental vitiligo while the generation of oxidative stress in non-segmental vitiligo may be attributed to the lower levels of glutathione peroxidase and reduced glutathione[31].

Inhibition of thioredoxin reductase, a free radical scavenger located in the membrane of melanocytes[32] also contributes to oxidative stress generation in vitiligo epidermis. Higher extracellular calcium levels result in increased superoxide radicals that in turn lead to the inhibition of tyrosinase[33]. Table 3 summarizes the sources of H_2O_2 documented to date in vitiligo pathogenesis.

Autoimmune hypothesis—It is based on studies that have demonstrated an association between vitiligo and other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease, alopecia areata etc., and also the presence of circulating antimelanocyte and antikeratinocyte antibodies in the sera of vitiligo patients.

Humoral immune response in vitiligo

Antibodies against melanocyte antigens are found in the sera of vitiligo patients mainly belonging to IgG class. Our results have also shown that 84% of vitiligo patients at Baroda exhibit antimelanocyte antibodies

Table 3—Sources for epidermal/systemic H_2O_2 generation in vitiligo

Source	Site of H_2O_2 generation/ accumulation	Increase/ Decrease
Monoamine oxidase A[18]	Epidermis	Increase
Superoxide dismutase[29]	Blood	Increase
Glucose 6 phosphate dehydrogenase[29]	Blood	Decrease
NADPH oxidase[99]	Epidermis	Increase
Photoxidation of pterins[100]	Epidermis	Increase
Nitric oxide synthases[101]	Epidermis	Increase
Short circuit in 6BH4 recycling[16]	Epidermis	Increase
Catalase[27,28]	Blood and epidermis	Decrease
Glutathione peroxidase and reduced glutathione[29]	Blood	Decrease
Tyrosinase related protein 1[102]	Epidermis	Decrease
Xanthine oxidase[103]	Blood	Increase

in their circulation[34]. The principal melanocyte antigens recognized by these antibodies are tyrosinase[35], gp100/pmel 17, (a melanosomal matrix glycoprotein), and tyrosinase related proteins 1 and 2 (TRP 1 and TRP 2)[36,37]. These cell differentiation antigens are localized primarily to melanosomes[12]. The transcription factors such as SOX9 and SOX10 also act as melanocyte autoantigens[38]. Autoantibodies against HLA Class I molecules are also reported in vitiligo[39]. A summary of the serum antibody reactive autoantigens implicated in vitiligo is given in Table 4. A positive correlation is observed between the level of melanocyte antibodies and the disease activity[40]. *In vitro* studies showed that antibodies derived from vitiligo patients are able to destroy melanocytes by complement mediated damage and antibody dependent cellular cytotoxicity (Fig. 3)[41]. Recently, a surface receptor, melanin concentrating hormone receptor 1 (MCHR 1) is detected as an autoantibody target in 16% vitiligo sera[42]. Circulating organ specific autoantibodies particularly to thyroid, adrenal and gastric glands are commonly detected in the sera of vitiligo patients[43].

Exact role of antimelanocyte antibodies in the pathogenesis of vitiligo remains unknown. Autoantibodies against pigment cells might result from a genetic predisposition to immune dysregulation at T cell level[44]. Alternatively, cross-reacting antigens expressed either on other target cells or infecting microorganisms could elicit autoantibody

Table 4—Antigens recognized by vitiligo autoantibodies

Autoantigens	Reference
Tyrosinase	Kemp *et al.* 1997[35]
TRP 2	Okamoto *et al.* 1998[104]
TRP 1	Kemp *et al.* 1998b[37]
Pmel 17	Kemp *et al.* 1998a[36]
MCHR 1	Kemp *et al.* 2002[42]
SOX 9	Hedstrand *et al.* 2001[38]
SOX 10	Hedstrand *et al.* 2001[38]

production. Vitiligo antibodies could also result from an immune response to melanocyte antigens released following damage to pigment cells by other mechanisms, and these antibodies might then exacerbate the condition. The selective destruction of melanocytes might be due to antibody reactivity directed to the antigens preferentially expressed on pigment cells[35] or from an antibody response against antigens expressed on a variety of cell types[45] that might selectively destroy melanocytes because they are intrinsically more sensitive to immune mediated injury than other cells[46].

Cell mediated immune response in vitiligo

Histopathological investigations of the perilesional skin of vitiligo have suggested the involvement of lymphocytes in depigmentation process. Immunohistochemical studies have confirmed the presence of infiltrating T cells[47]. T cell infiltrates with a predominant presence of CD8+ T cells are detected in generalized vitiligo[48]. Autoimmune diseases are often associated with an expansion of peripheral CD4+ T cells[49] and an elevated CD4+/CD8+ ratio is reported[50] in patients with stable vitiligo as well as in their first-degree relatives. Nevertheless, a decrease in CD4+ T cell population along with a reduced CD4+/CD8+ ratio is also observed[51]. A substantial number of infiltrating T cells express the cutaneous lymphocyte antigen-CLA[52], typical of skin homing T cells and a recent study has shown CLA positive cytotoxic T cells in perilesional skin[48]. Melan A/Mart1 (a melanosomal antigen) specific CD8+ T lymphocytes are also present in peripheral blood of vitiligo patients[53]. Interestingly, Melan-A/Mart 1 specific CD8+ T cells are identified in the inflammatory lesions of melanocyte destruction followed by infusion of Melan-A/Mart 1 specific CD8+ T cell clones in melanoma patients[54]. The above findings suggest a direct evidence for the T cell

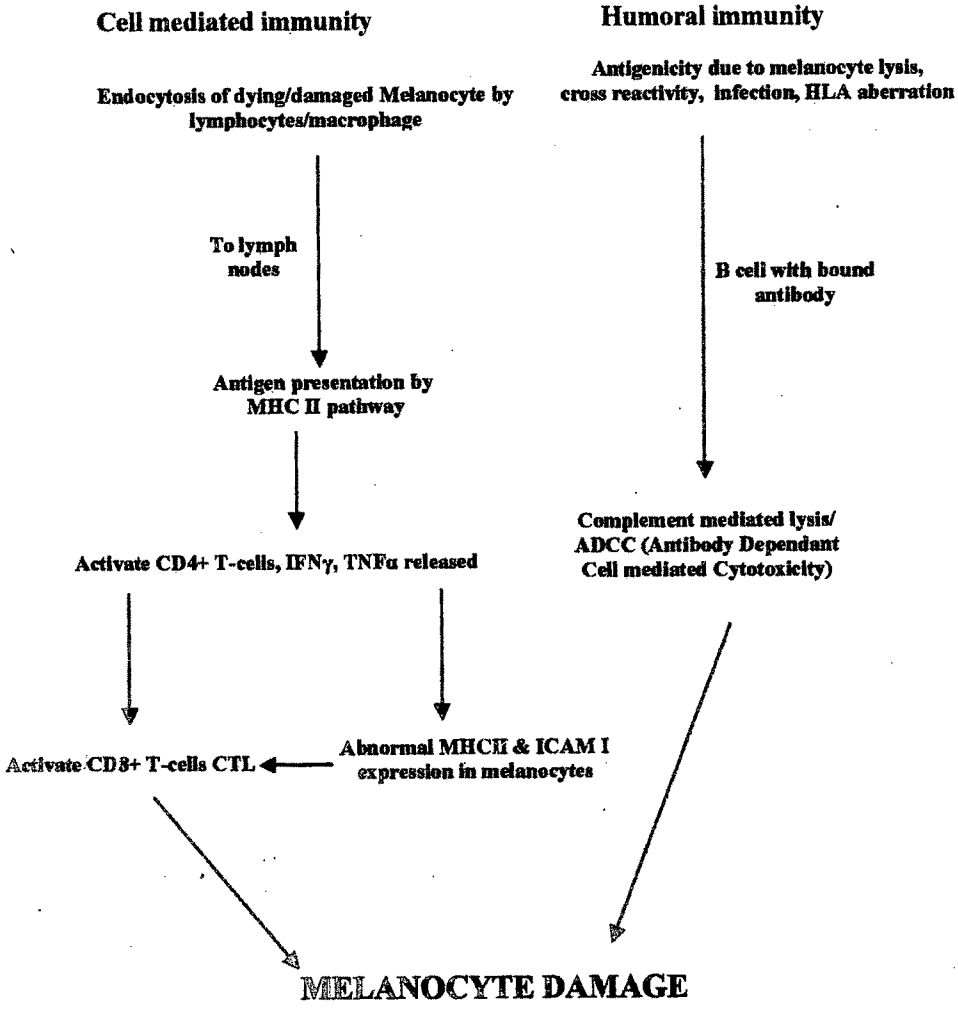

Fig. 3—Sumamry of the cellular and humoral immune mechanisms in vitiligo. Melanocyte damage can be caused by T cell mediated cytotoxicity or humoral immune response. Intrinsic and extrinsic melanocyte damage leads to lysis of melanocytes. The released content can be presented by MHC II pathway leading to the activation of T helper cells which further leads to the activation of CD8+T cells resulting in cell cytotoxicity. Autoantibodies against melanocyte proteins result in antigen-antibody complex formation leading to complement activation or ADCC (Antibody Mediated Cell Cytotoxicity).

mediated melanocyte destruction in vitiligo patients. However, lymphokine-activated cytotoxicity is reported to be normal in patients with progressive vitiligo[55].

Immunohistochemical studies of the perilesional area of generalized vitiligo patients mainly detect CD4+ and CD8+ T cells in the infiltrate. These cells activate the expression of molecules such as interleukin 2 receptor (IL 2R and CD25), interferon γ (IFN γ), which further enhance T cell trafficking to the skin by increasing ICAM-I expression[52]. In parallel and supposedly in correlation with these local findings, activation of circulating T lymphocytes is also observed. In non-segmental vitiligo, increased expression of CD25 and or HLA DR[56], elevated CD45RO memory T cells[57] and decreased CD45RA+ naive subsets are demonstrated[56], although the latter observation has not been confirmed by other studies[58]. *In vitro* studies have demonstrated an increased production of proinflammatory cytokines-IL-6 and IL-8 by monocytes of patients with active vitiligo. IL-6 and IL-8 not only play an important role in effector cell migration and effector target attachment, but also cause B cell activation (Fig. 3)[59]. Activation of T cell mediated immune response has been confirmed in vitiligo by detecting significantly increased levels of

soluble interleukin 2 receptors (SIL-2R), especially in generalized, focal and segmental types of vitiligo[60].

Vitiligo and apoptosis

Exact pathway of destruction of melanocytes in vitiligo patients is not yet known, however, apoptotic type of cell death has been suggested[61]. Cytokines such as IL I, IFN γ or tumor necrosis factor α (TNFα) that are released by lymphocytes, keratinocytes and melanocytes can initiate apoptosis[61]. Recently an imbalance of cytokines in the epidermal microenvironment of lesional skin is reported which could impair the normal life and function of melanocytes. The observed increased levels of TNFα, a paracrine inhibitor of melanocytes could be leading to its death[62]. Activated cytotoxic T lymphocytes can also induce apoptosis through the perforin/granzyme or Fas/Fas ligand pathway. The regulatory molecules of apoptosis[63] seem to be well expressed in vitiliginous melanocytes and it is shown that relative apoptotic susceptibility of vitiligo melanocytes is comparable with that of normal melanocytes[48].

Convergence theory

Several hypotheses on the mechanism of pathogenesis have been combined and formulated a convergence theory to explain the etiopathogenesis of vitiligo[2]. According to this theory stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation may contribute to vitiligo pathogenesis in varying proportions.

Genetic polymorphism: Candidate genes associated with vitiligo susceptibility

The complex genetics of vitiligo involves multiple susceptibility loci, genetic heterogeneity and incomplete penetrance with gene-gene and gene-environment interactions[64]. A few genes that are reported to contribute to vitiligo susceptibility are shown in Table 5 and are described below.

Autoimmune regulator I gene (AIRE I)—Vitiligo is commonly associated with autoimmune polyglandular syndrome type I (APS I)[65]. APS I associated vitiligo is proposed to be a consequence of immune dysregulation resulting from mutations in *AIRE* I gene. *AIRE* gene product is a transcription factor and is normally expressed in immune related organs such as thymus and lymph nodes[66]. Mutational analysis identified two mutations in this gene in Swiss and Finnish APS I patients[65,66]. Homozygous or heterozygous mutations of *AIRE I* are also implicated in autoimmune Addison's disease in the context of APS I[67].

Table 5—Genes that contribute to vitiligo susceptibility

Gene	Reference
AIRE	Nagamine *et al.* 1997[66]
CTLA4	Blomhoff *et al.* 2005[69]
CAT	Casp *et al.* 2002[70]
COMT	Tursen *et al.* 2002[73]
LMP and *TAP*	Casp *et al.* 2003[75]
MC1R and *ASIP*	Na *et al.* 2003[80]
Angiotensin converting enzyme gene	Jin *et al.* 2004[83]
Estrogen receptor gene	Jin *et al.* 2004[85]
Lymphoid protein tyrosine phosphatase (*PTPN 22*)	Canton *et al.* 2005[89]

Cytotoxic T lymphocyte antigen 4 (CTLA 4)—CTLA 4 is a T cell surface molecule involved in T cell apoptosis and regulation of T cell activation[68]. It is, therefore, a candidate gene for contributing to the development of T cell mediated autoimmune disease if its expression or function is adversely affected by the mutations resulting in polymorphic alleles. Studies suggest that vitiligo when not associated with an autoimmune disorder is not influenced by *CTLA 4* polymorphism[69].

Catalase (CAT)—*CAT* gene is identified as a candidate gene as reduction of catalase activity results in accumulation of H_2O_2 in epidermis of vitiligo patients[26]. An association is established between vitiligo and a single nucleotide polymorphism (SNP) in exon 9 of *CAT* gene[70] as it has been observed that T/C heterozygocity is more frequent among vitiligo patients than in controls. C allele is transmitted more frequently to patients than to controls suggesting that linked mutations in or near *CAT* gene may contribute to a quantitative deficiency of catalase activity in vitiligo patients.

Catechol-o-methyl transferase (COMT)—COMT is a ubiquitous enzyme catalyzing the *o*-methylation of biologically active or toxic catechols, and plays a major role in the metabolism of drugs and neurotransmitters[71]. In melanocytes, COMT can prevent the formation of toxic *o*-quinones during melanin synthesis[72]. It has been found that the epidermal homogenates from vitiligo patients expressed higher levels of COMT activity compared to controls[19]. *COMT* polymorphism is not detected in

all types of vitiligo, however, *COMT*- LL (low activity homozygote) genotype is significantly associated with acrofacial vitiligo[73].

Light molecular weight protein (LMP) and transporter associated protein (TAP)—Genes within class II region of the major histocompatibility complex (MHC) are associated with several autoimmune diseases[74]. This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight protein polypeptide 2 and 7 (LMP 2 and 7) and transporter associated with antigen processing protein 1 (TAP 1). LMP 2 and LMP 7 are involved in degradation of ubiquitin tagged cytoplasmic proteins into peptides, whereas TAP 1 is involved in transportation of peptides into endoplasmic reticulum, where they are exposed to nascent MHC class I molecules[74]. A genetic association of early onset of vitiligo is observed with *TAP 1* gene[75]. Moreover alleles from heterozygous parents are disequillibratedly transmitted to affected offspring for *TAP 1* gene, as well as for closely linked *LMP 2* and *LMP 7* genes.

Melanocortin 1 receptor (MC1R) and agouti signaling protein (ASIP) genes—The melanocortin I receptor (*MC 1 R*) gene codes for melanocyte stimulating hormone receptor (MSHR) and a number of loss of function mutations in *MC 1 R* are reported[76]. *MC 1 R* is as a major determinant of sun sensitivity[77]. Melanogenesis is regulated by the binding of α melanocyte-stimulating hormone (α MSH) and of ASIP (Agouti Signaling Protein) to MSHR. Binding of ASIP to MSHR precludes α MSH initiated signaling and thus blocks production of cAMP, leading to down-regulation of eumelanogenesis[78]. *MC 1R* mRNA expression is also down regulated by ASIP[79]. Studies on polymorphism in *MC 1R* and *ASIP* have revealed that Val92Met (G274A) and Arg163Gln (A488G) represent the most common SNPs of *MC 1R* in Korean population. Frequency and carriage rate of G274A allele of *MC 1R* and g.8818A>G allele of *ASIP* is higher in vitiligo patients. The patients who carry both SNPs of *MC1R* and *ASIP* are prone to vitiligo disease[80].

Angiotensin converting enzyme gene (ACE)—Studies have shown an association of *ACE* gene I/D polymorphism in intron 16 and autoimmune diseases[81]. Angiotensin converting enzyme is capable of inactivating bradykinin, modulating cutaneous neurogenic inflammation and degrading substance P and other neuropeptides[82]. The *ACE* genotype distribution and allelic frequencies are significantly different between vitiligo patients and controls, suggesting a strong association of *ACE* gene polymorphism with vitiligo[83].

Estrogen receptor gene (ESR I)—It is reported that high estrogen levels in serum is associated with increase in skin pigmentation and successful treatment of vitiligo is possible with estrogen[84]. Association of *ESR I* gene and vitiligo shows that *ESR I* intron 1 C/T polymorphism is associated with female or generalized vitiligo[85].

Lymphoid protein tyrosine phosphatase (PTPN 22) gene—PTPN 22 gene encodes lymphoid protein tyrosine phosphatase (LYP), which is important in negative control of T lymphocyte activation[86]. Lymphoid protein tyrosine phosphatase is expressed in T lymphocytes and associates with C-terminal Src kinase (CSK) to form a complex that suppresses T cell receptor signalling kinases LCK and FYN[87]. The missense R620W polymorphism in *PTPN22* gene at the nucleotide 1858 (1858 C > T) in codon 620 (620 Arg >Trp) has been associated with autoimmune diseases[88]. The disease associated *LYP* variant Trp620 prevents the interaction of LYP with CSK[88]. Consequently, the T cell receptor associated kinases might exhibit an uncontrolled T cells induction, and this may increase the overall reactivity of the immune system and predispose an individual to autoimmune diseases. Studies on *PTPN22* gene show that 1858T allele is significantly over represented in vitiligo patients compared to controls, indicating *LYP* missense R620W polymorphism may have an influence on the development of generalized vitiligo, which further provides evidence for the autoimmunity as an etiological factor[89].

Linkage and association studies

Familial clustering and linkage disequilibrium studies suggest that genetic factors predispose vitiligo, although a clear transmission pattern and cosegregation of vitiligo with specific mutations have not been demonstrated[90].

HLA associations—The frequent association of vitiligo with other autoimmune diseases suggests an association between HLA system and vitiligo predisposition. The HLA loci are strongly linked to other loci in the major histocompatibility region of chromosome 6p. Therefore, vitiligo associated HLA alleles may not be disease specific, but are the genetic

markers that usually co-inherit in the population (i.e. in strong linkage disequilibrium) with the actual disease allele at another locus within the major histocompatibility region[105]. Linkage disequilibrium studies in different populations have consistently showed a significant association between HLA system and vitiligo predisposition. A case control study carried out in Kuwaiti population showed that allele frequencies of HLA B21, Cw6 and DR53 are increased significantly, whereas the frequencies of HLA A19 and DR52 are significantly decreased in vitiligo patients[91]. Significant increase in frequencies of HLA A30, Cw6, and DQw3 and significant decrease in frequency of CQA40 has been reported in Northern Italian vitiligo patients[92]. In Dutch patients, a family based case control association and linkage disequilibrium analyses have shown linkage and association to DRB4*0101 and DQB1*0303 alleles with vitiligo susceptibility[93]. A complex segregation analysis and linkage disequilibrium analyses with respect to microsatellite loci spanning, the HLA conducted in a set of 56 multi generation families has shown that the penetrance and risk estimations discriminated two sets of vitiligo patients: those with an early onset of vitiligo exhibit cosegregation with a dominant mode of inheritance without environmental effects and those with late onset of vitiligo exhibit cosegregation with recessive genotype and being influenced by environmental effects. It has also shown significant linkage disequilibrium between loci D6S276 and D6S273 in vitiligo patients compared to controls is also reported[90]. HLA association studies reported in vitiligo are shown in Table 6.

Genome wide linkage analyses—Genome wide linkage scans involve typing of families using polymorphic markers that are positioned across the whole genome, followed by calculating the degree of linkage of the marker to a disease trait. Positional candidate genes can be identified by examining the regions around the peaks of linkage. Several genome wide linkage analyses of vitiligo have been performed in recent years and multiple linkages to vitiligo are identified[94-97]. A study involving 16 European American pedigrees with cosegregation of systemic lupus erythematosus (SLE) and vitiligo, identified a potentially informative genomic region at 17p13, which may contain the putative gene *SLEVI* for vitiligo related SLE. A highly suggestive linkage between vitiligo and a locus in chromosome segment 1p31.3-p32.2 termed *AIS1* is reported in a single large white family[96]. Another genome wide linkage scan in 71 white multiplex families with vitiligo from North America and UK have confirmed the linkage of *AIS1* with vitiligo establishing its importance as a major

Table 6—HLA associations reported in vitiligo

Positive association	Negative association	Reference
DRB1*04-DQB1*0301	DRB1*15-DQB1*0602	Fain *et al.*, 2006 [106]
DQA1*0302, *0601, DQB1*0303, *0503	*0503 DQA1*0501	Yang *et al.*, 2005 [107]
A*2501, A*30, B*13, B*27, Cw*0602	A*66	Zhang *et al.*, 2004 [105]
DR4, DR53	DR3	de Vijlder *et al.*, 2004 [108]
DR3, DR4, DR7	---	Tastan *et al.*, 2004 [109]
DRB4*0101, DQB1*0303	---	Zamani *et al.*, 2001 [93]
DRB1*0701, DQB1*0201, DPB1*1601	---	Buc *et al.*, 1998 [110]
A2, A10, A30 + A31, B13, B15	A28, B46	Wang *et al.*, 2000 [111]
A2, Dw7	---	Buc *et al.*, 1996 [112]
B21, Cw6, DR53	A19, DR52	Al-Fouzan *et al.*, 1995[91]
DR6	DQ2	Valsecchi *et al.*, 1995 [113]
Bw6, DR7	---	Venkataram *et al.*, 1995 [114]
DR6	Cw7	Venneker *et al.*, 1993 [115]
B46, A31, Cw4	---	Ando *et al.*, 1993 [116]
DR12, A2	---	Schallreuter *et al.*, 1993 [117]
A30, Cw6, DQ3	C4AQ0	Orecchia *et al.*, 1992[92]
DR1	---	Poloy *et al.*, 1991 [118]
A30, Cw6, B27, DR7	DR1, DR3	Finco *et al.*, 1991 [119]
A2, A3	---	Dai *et al.*, 1990 [120]
DR4, DQ3	---	Dunston and Halder, 1990 [121]
DR4	---	Foley *et al.*, 1983 [122]
BW35	---	Metzker *et al.*, 1980[123]
A1, A2, A31	A10	Kachru *et al.*, 1978 [124]

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Letter to the Editor
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Antioxidant status of segmental and non-segmental vitiligo

EM Shajil and Rasheedunnisa Begum*

Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India
*Address correspondence to Rasheedunnisa Begum, e-mail: rasheeda@icenet.co.in

Sir,

Vitiligo is a depigmenting disorder resulting from the loss of melanocytes in the skin and affects 1–2% of the world population. Several hypotheses are proposed about the pathogenesis of vitiligo and oxidative stress hypothesis considers a systemic involvement during the course of the disease. We have earlier reported impairment of systemic antioxidant status of Baroda vitiligo patients (Agrawal et al., 2004) and we now show analysis of the blood antioxidant status of segmental and non-segmental clinical types of vitiligo.

Blood samples were collected from 124 vitiligo patients without any other associated diseases and also from 126 age and sex matched healthy consenting volunteers without any disease for the estimation of antioxidant parameters. Vitiligo patients were further classified into segmental and non-segmental vitiligo. We have analyzed the blood antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx); non-enzymatic antioxidants like reduced glutathione (GSH), vitamin E; erythrocyte lipid peroxidation levels (LPO) and glucose-6-phosphate dehydrogenase (G6PDH) activity in segmental and non-segmental vitiligo patients and controls according to the standard established methods (Agrawal et al., 2004) and the results are shown in Table 1. A significant increase in erythrocyte SOD activity was observed in both the types of vitiligo patients compared with controls (Table 1). However, no significant change was observed in segmental vitiligo compared with non-segmental vitiligo. There are no specific reports available on the SOD levels in different clinical types of vitiligo. However, an increase in SOD activity was reported (Chakraborty et al., 1996; Agrawal et al., 2004). Catalase activity was found to be significantly lower in segmental vitiligo, while no significant change in the catalase activity was observed in non-segmental vitiligo. Also, no significant change was observed in segmental vitiligo compared to non-segmental vitiligo (Table 1). There is no report on the catalase levels in different clinical types of vitiligo. No significant change in the erythrocyte catalase activity was reported (Beazley et al., 1999). A significant decrease in GPx levels was observed in non-segmental and no change was observed in segmental vitiligo. Segmental vitiligo showed no significant change compared with non-segmental vitiligo (Table 1). Beazley et al. (1999) reported low levels of GPx in the blood of generalized vitiligo patients compared with controls and there are no reports on other clinical types of vitiligo. We observed significant decrease in G6PDH activity in both the types of vitiligo, however, no significant change is observed in segmental vitiligo compared with non-segmental vitiligo (Table 1). There is no report on the G6PDH levels in different clinical types of vitiligo. However, Agrawal et al. (2004) and Saha et al. (1982) reported a decrease in G6PDH levels in vitiligo patients compared with controls. Significantly, lower GSH levels were observed non-segmental vitiligo compared with controls, however, no change is observed in segmental vitiligo compared with non-segmental vitiligo (Table 1). There are no reports on the GSH levels in different clinical types of vitiligo. Passi et al. (1998) showed that epidermal GSH levels of active vitiligo patients were significantly lower compared with controls. Agrawal et al. (2004) and Yildirim et al. (2003) have also shown a significant decrease in the erythrocyte GSH levels in non-segmental vitiligo patients compared with controls. However, Picardo et al. (1994) found no change in the erythrocyte GSH activity of vitiligo patients. Changes in plasma vitamin E levels were non-significant in both the types of vitiligo and among segmental and non-segmental vitiligo (Table 1). There are no reports on the vitamin E levels in different clinical types of vitiligo. Agrawal et al. (2004) and Picardo et al. (1994) showed no change in the plasma vitamin E levels in vitiligo patients compared to controls. Significant increase in LPO levels was observed in both the clinical types of vitiligo, however, no change is observed in segmental vitiligo compared to non-segmental vitiligo (Table 1). Non-significant changes in plasma lipoperoxides of vitiligo patients were reported by Picardo et al. (1994).

From the above, it is clear that the status of enzymatic and non-enzymatic antioxidants is altered in both the clinical types of vitiligo compared with controls. Increased levels of erythrocyte SOD could enhance the systemic production of H_2O_2. The downstream antioxidant enzymes that neutralize H_2O_2, i.e. catalase, is found

Table 1. Antioxidant status of different clinical types of vitiligo compared with controls. Results in vitiligo patients and controls were compared using the paired Student's *t*-test. One-way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between different groups.

Type of vitiligo	Antioxidant parameters[a]						
	SOD	CAT	GPx	G6PDH	GSH	VIT E	LPO
Control (n = 126)	1830 ± 51.33	266.4 ± 4.59	17.28 ± 0.40	14.14 ± 0.48	34.47 ± 0.66	7.913 ± 0.1709	184.5 ± 2.71
Non-segmental (n = 94)	2885 ± 55.41***	252.0 ± 6.450 NS	15.89 ± 0.2611*	7.444 ± 0.3033***	31.52 ± 0.7020**	7.654 ± 0.1873 NS	201.8 ± 3.769***
Segmental (n = 30)	2892 ± 99.75***	233.7 ± 11.22**	15.28 ± 0.5578 NS	7.712 ± 0.5223***	31.99 ± 1.268 NS	7.718 ± 0.3359 NS	204.9 ± 5.518 **

SOD is expressed as U/g Hb; CAT as k/g Hb; GPx as μmoles of GSH utilized/s/g Hb; G6PDH as mmoles of nicotinamide adenine dinucleotide phosphate (NADP) reduced to NADPH/min/g Hb; GSH as mg GSH/dl blood; Vitamin E as μg Vit E/ml of plasma and LPO is expressed as nmoles of malonaldehyde formed/g Hb.

NS, non-significant; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione; G6PDH, glucose-6-phosphate dehydrogenase; LPO, lipid peroxidation levels; GPx, glutathione peroxidase.

[a]Values are expressed as mean ± SE.

*P < 0.05; **P < 0.02, ***P < 0.01.

to be significantly decreased in segmental vitiligo patients, whereas significantly lowered GPx and GSH levels are found in non-segmental vitiligo patients. Plasma vitamin E levels of these patients remain unchanged, however, with significantly lower nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) levels (because of reduced levels of G6PDH), the non-enzymatic cycle may not proceed to completion. Hence, the free radicals may accumulate and contribute to the build up of oxidative stress in the system. Thus high levels of SOD, low levels of catalase and G6PDH could contribute to oxidative stress in segmental vitiligo; while high levels of SOD, low levels of GPx, G6PDH and GSH could contribute to oxidative stress in non-segmental vitiligo as is evident by high LPO levels in vitiligo patients. Elevated LPO levels in both the clinical types of vitiligo (Table 1) suggest that systemic oxidative stress is the hallmark feature of all these patients. Nevertheless, there are minor changes in the antioxidant status among different clinical types of vitiligo. The difference between the generation of oxidative stress in segmental and non-segmental seems to lie in the levels of catalase and GPx. The low levels of catalase may contribute for the generation of oxidative stress in segmental vitiligo while the generation of oxidative stress in non-segmental vitiligo may be attributed to the lower levels of GPx and GSH. In conclusion, impairment of the systemic antioxidant system resulting in oxidative stress in both the clinical types of vitiligo is observed in this study indicating that melanocyte damage in vitiligo may be linked to generalized oxidative stress.

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Original Research Article

Study on the Antioxidant Status of Vitiligo Patients of Different Age Groups in Baroda

DEEPALI AGRAWAL[1], E. M. SHAJIL[1], Y. S. MARFATIA[2] and RASHEEDUNNISA BEGUM[1]

[1]*Department of Biochemistry, Faculty of Science, M.S. University of Baroda, Vadodara, Gujarat, India;* [2]*Department of Skin and VD, Sir Sayaji Rao Gaikwad Medical College, Vadodara, Gujarat, India*
**Address reprint request to Rasheedunnisa Begum, Department of Biochemistry, Faculty of Science, M.S. University of Baroda, Vadodara, Gujarat 390 002 India. E-mail: rasheeda@wilnetonline.net*

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One of the major hypotheses in the pathogenesis of vitiligo is the oxidative stress hypothesis. Pollution plays a major role in the production of free radicals. Gujarat, a highly industrialized state in India has a high prevalence of vitiligo patients. No previous studies were done on the age-dependent antioxidant status of vitiligo patients in Baroda city, Gujarat. Blood samples were collected from vitiligo patients of different age groups (5–15, 16–25, 26–35, 36–45 yr) and from age matched healthy volunteers. Antioxidant enzymes in blood such as catalase, superoxide dismutase, glutathione peroxidase and non-enzymatic antioxidants such as reduced glutathione and plasma vitamin E were estimated. Lipid peroxidation levels in erythrocytes and the reducing equivalent system, i.e. glucose-6-phosphate dehydrogenase were also measured. Significant increase in superoxide dismutase activity and lipid peroxidation levels in erythrocytes was observed in all age groups of vitiligo patients as compared with age-matched healthy controls, wherein an increase of 55% ($P < 0.02$) was observed in superoxide dismutase activity and lipid peroxidation levels in 36–45 yr age group. Whole blood glutathione levels, erythrocyte glutathione peroxidase and glucose-6-phosphate dehydrogenase activity were decreased significantly, whereas erythrocyte catalase activity and plasma vitamin E levels were not different in vitiligo patients as compared with age-matched healthy controls. No specific age group showed a significant difference. This is the first report on the age-dependent antioxidant status of vitiligo patients in Baroda. The disease affects individuals of any age group as shown in this study and systemic oxidative stress might precipitate the pathogenesis of vitiligo in susceptible patients.

Key words: Age-dependent, Systemic oxidative stress, Catalase, Superoxide dismutase, Glutathione peroxidase

INTRODUCTION

Vitiligo is an idiopathic, acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches of different size and shape; it affects 1–2% of the world population (1, 2). Based on a few dermatological outpatient records, the incidence of vitiligo is found to be 0.5–2.5% in India (3, 4). Gujarat and Rajasthan states have the highest prevalence ~8.8% (5). Vitiligo may be restricted to a limited cutaneous territory (segmental vitiligo) or generalized in symmetric patches (6). The active or stable phase of vitiligo is defined on the basis of the progression or appearance of lesions in the last 3 months and the absence of new lesions or their progression in the last 6 months, respectively (7). Although vitiligo is extensively addressed in the past five decades, its etiology is still being debated (6, 8–13).

Several hypotheses have been proposed about the pathogenesis of vitiligo and the oxidative stress hypothesis considers a systemic involvement in the course of the disease (14, 15). Oxidative stress could act as the initial triggering event in melanocyte degeneration. In accordance with this view, the present study was undertaken and we have selected Baroda for our study, as it is one of the highly industrialized cities in Gujarat. We have determined the status of blood antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx); non-enzymatic antioxidant molecules like reduced glutathione (GSH),

Abbreviations – CAT, catalase; G6PDH, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, reduced glutathione; LPO, lipid peroxidation; ROS, reactive oxygen species; SOD, superoxide dismutase

vitamin E; erythrocyte lipid peroxidation (LPO) levels and glucose-6-phosphate dehydrogenase (G6PDH) activity in different age groups of Baroda vitiliginous patients.

MATERIALS AND METHODS

Patients and Controls

For the estimation of antioxidant parameters in the blood, 63 vitiligo patients were divided into different age groups (5–15, 16–25, 26–35 and 36–45 yr) after written informed consent had been obtained. The patients had no associated diseases. Sixty controls were age matched healthy consenting volunteers.

Estimation of Blood Antioxidant Parameters

Antioxidant enzyme activity, non-enzymatic antioxidants and oxidative damage of polyunsaturated fatty acids (LPO) in vitiligo patients and in healthy controls were standardized and estimated according to the established methods.

Erythrocyte SOD was assayed according to the method of Nishikimi *et al.* (16). Nitroblue tetrazolium salt (NBT), a superoxide radical scavenger is reduced by superoxide to form blue formazan whose absorption was measured at 560 nm. One unit of SOD is defined as the amount of enzyme required to give 50% inhibition of the NBT reduction reaction compared with enzyme control.

Erythrocyte CAT activity in the hemolysate was assayed by the method of Aebi (17). The rapid decomposition of H_2O_2 to water was directly assayed by measuring the decrease in absorbance at 240 nm. CAT activity is expressed in terms of k/g hemoglobin/s, where k is the velocity constant of the decomposition of H_2O_2 to water.

Erythrocyte GPx was assayed according to the method of Beutler et al. (18). GPx acts on H_2O_2 in the presence of GSH thereby depleting it, and the remaining GSH is measured by the DTNB color reaction read at 412 nm. GPx activity is expressed in terms of μmol GSH utilized/g hemoglobin/s.

Total GSH in the whole blood was determined by the method of Beutler et al. (19). DTNB, a disulfide compound is readily reduced by sulfhydryl compounds forming a yellow colored chromophore whose absorbance was measured at 412 nm. The results are expressed in mg of GSH/dl of blood.

The plasma vitamin E level was estimated by the method of Hansen and Warwick (20). Vitamin E was extracted from the plasma sample with hexane and the levels were monitored spectrofluorometrically at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. The concentration was calculated from the standard graph of vitamin E and the results are expressed in μg vitamin E/ml of plasma.

Erythrocyte G6PDH was assayed by the method of Korenberg and Horecker (21). The increase in the absorbance at 340 nm, due to the reduction of NADP to NADPH was measured. The activity of the enzyme is expressed in terms of mmoles of NADP reduced to NADPH/g hemoglobin/min.

Erythrocyte LPO was estimated according to the procedure of Beuge and Aust (22). Malonaldehyde produced during peroxidation of lipids served as an index of LPO. Methylenedioxyamphetamine (MDA) reacts with thiobarbituric acid to generate a colored product, which was measured at 532 nm and the results are expressed as nmol MDA formed/g hemoglobin/min.

Statistical Analysis

Results in vitiligo patients and in age-matched controls were compared using the paired student's t-test. One way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between different age groups of vitiligo patients utilizing statistical software program Prism. $P \leq 0.05$ is considered significant.

RESULTS

Vitiligo is a universal disease. The present study has been attempted to find out whether age has any relevance for the onset of the disease. Vitiligo patients were divided into four age groups i.e. 5–15, 16–25, 26–35 and 36–45 yr for the analysis of the antioxidant status. The age groups 5–15, 16–25, 26–35 and 36–45 yr showed an increase in the erythrocyte SOD activity of 44, 29, 26 and 55%, respectively, compared with the age matched controls (Fig. 1). Similarly an increase in the LPO levels of the vitiliginous patients was observed, i.e. 25, 21, 41 and 55%, respectively, in the respective age groups compared with their controls (Fig. 2). However, a significant decrease in GPx activity, i.e. 33.3, 26.3, 37.7 and 31.63% was observed in the respective age groups compared with their controls (Fig. 3). A significant decline in G6PDH activity was also observed, i.e. of 10, 10.2, 18 and 24%, respectively, in the respective age groups (Fig. 4). A significant decrease in whole blood GSH levels, i.e. 20% in 5–15 and 16–25 yr, 23% in 26–35 yr and 18% in 36–45 yr age groups was

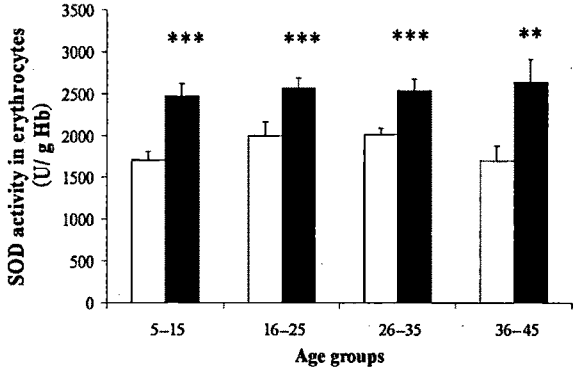

Fig. 1. SOD activity in erythrocytes of controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients. **P < 0.02; ***P < 0.01.

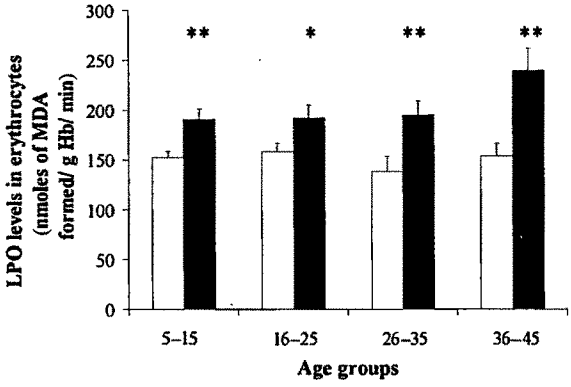

Fig. 2. Erythrocyte LPO levels in controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients. *P < 0.05; **P < 0.02.

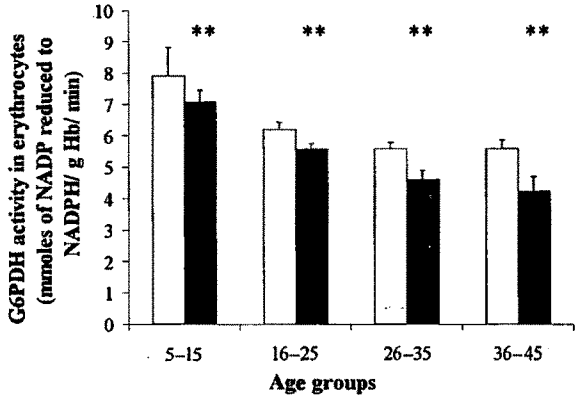

Fig. 4. G6PDH activity in erythrocytes of controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients. **P < 0.02.

observed compared with their controls (Fig. 5). Erythrocyte catalase and plasma vitamin E levels showed no significant change in the vitiliginous patients compared with their controls (Figs 6 and 7).

Further, our analysis by ANOVA suggests that vitiligo might develop at any age and that all age groups are equally susceptible to this disease. The age group 16–25 yr that has more patients (29 patients) compared with the other age groups was divided into segmental and non-segmental types of vitiligo (Table 1). They are further subdivided into active non-segmental vitiligo, active segmental vitiligo, stable non-segmental vitiligo, stable segmental vitiligo (Table 2) and analyzed for their antioxidant status. Interestingly, no significant difference was observed in the antioxidant status of the patients of segmental vs. non-segmental, active non-segmental vs. active segmental and stable non-segmental vs. stable segmental vitiligo.

DISCUSSION

Oxidative stress acting as the triggering event in melanocyte degeneration in the etiopathogenesis of vitiligo is well established (14, 23). Oxidative stress hypothesis is based on the fact that during melanin biosynthesis some intermediates are generated, such as 3,4-dihydroxyphenylalanine (dopa),

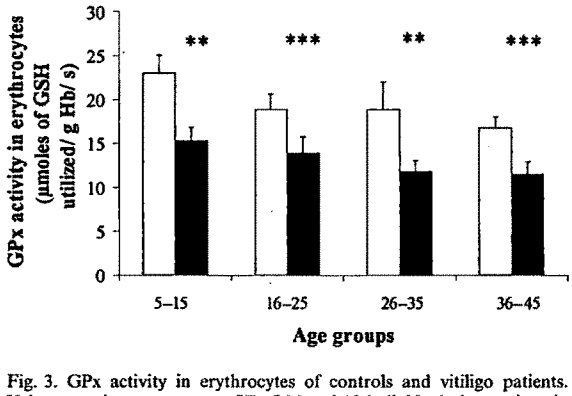

Fig. 3. GPx activity in erythrocytes of controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients. **P < 0.02; ***P < 0.01.

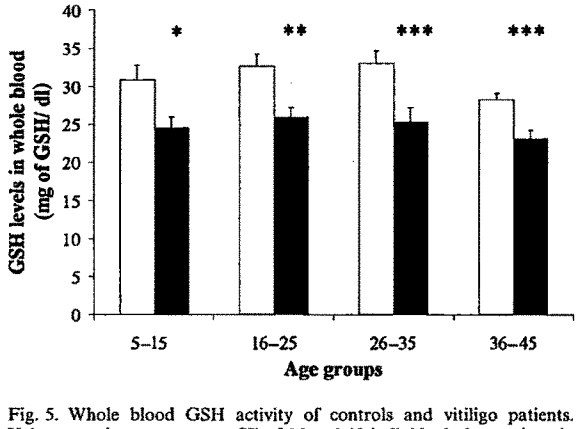

Fig. 5. Whole blood GSH activity of controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients. *P < 0.05; **P < 0.02; ***P < 0.01.

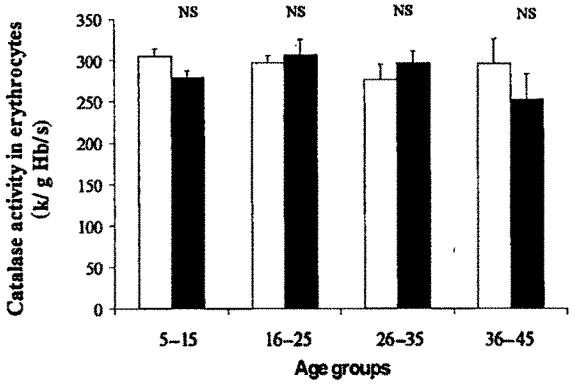

Fig. 6. CAT activity in erythrocytes of control and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients.

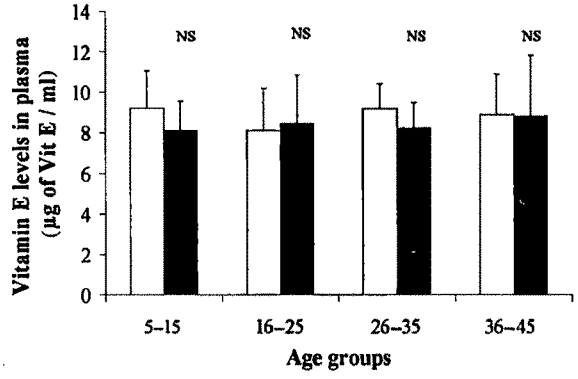

Fig. 7. Vitamin E activity in the plasma of controls and vitiligo patients. Values are given as mean ± SE of 15 and 12 individual observations in controls and patients in the 5–15 yr age group; 19 and 29 individual observations in controls and patients in the 16–25 yr age group; 14 and 11 individual observations in controls and patients in the 26–35 yr age group and 12 and 11 individual observations in controls and patients in the 36–45 yr age group, respectively. Empty bars are of controls and the filled bars are of vitiligo patients.

dopachrome and 5,6-dihydroxyindole (DHI), that are known to be toxic to melanocytes (24). Defective calcium homeostasis is also observed in vitiligo patients, that in turn affects the redox status of 6-biopterin/tetrahydrobiopterin equilibrium, monoamine oxidase activity among others (25–29). The final result of all these changes leads to the accumulation of H_2O_2, which further inhibits the CAT activity resulting in the destruction of melanocytes. Low epidermal CAT levels in both lesional and non-lesional epidermis of patients with vitiligo suggests that the entire epidermis may be involved in this disorder (30). The cytotoxic effect of reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals on melanocytes was studied (31, 32). Independent studies also showed that melanogenesis produces significant levels of ROS (33). The present study throws light on the environmental factors that could account for the systemic origin of ROS in the susceptible patients.

The SOD scavenges superoxide radicals and reduces their toxicity (34). In the present study, a significant increase in erythrocyte SOD activity was observed in all age groups of vitiligo patients compared with their respective controls (Fig. 1). The 36–45 yr age group showed the highest increase (55.15%) in SOD activity compared with other groups, however, this was not significant when ANOVA was applied. The increase in SOD activity in vitiligo patients was similar to the observations made by Chakraborty et al. (35). However, Picardo et al. (14) reported that SOD activity in erythrocytes of vitiligo patients was not significantly different from healthy age-matched controls. There was no significant difference in catalase activity (Fig. 6), whereas a significant decrease in GPx activity was observed in all age groups of vitiligo patients compared with the controls (Fig. 3). This was in accordance with the report of previous workers (7, 36). However, studies in vitiliginous melanocytes by other workers showed lower CAT activity (37, 38). GPx also catalyzes the reduction of hydroperoxides in the presence of GSH to form glutathione disulfide (39, 40).

We observed a significant decrease in G6PDH activity (Fig. 4) in concordance with Saha et al. (41). G6PDH is the first rate-limiting enzyme in the hexose monophosphate shunt pathway, playing an important role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH maintains glutathione in its reduced form, which is essential for the detoxification of reactive free radicals, lipid hydroperoxides and toxic compounds of endogenous and exogenous origin. In red cells, the

Table 1. Systemic antioxidant status of non-segmental vs. segmental vitiligo patients in 16–25 yr of age group

	Parameters						
Type of vitiligo	SOD	CAT	GPx	G6PDH	GSH	Vit E	LPO
Non-segmental (n = 21)	2845 ± 201.64	296 ± 2.31	15.52 ± 1.17	5.70 ± 0.21	24.25 ± 0.96	8.00 ± 1.40	210 ± 13.20
Segmental (n = 8)	2515 ± 156.27	288 ± 12.60	13.23 ± 1.23	5.48 ± 0.60	24.25 ± 1.13	8.80 ± 1.90	205 ± 29.27

Values are expressed as mean ± SE.
SOD is expressed as U/g Hb; CAT is expressed as *k*/g Hb/s; GPx is expressed as µmol GSH utilized/g Hb/s; G6PDH is expressed as mmol NADP reduced to NADPH/g Hb/min; GSH is expressed as mg GSH/dl of blood; vitamin E is expressed in µg vit E/ml of plasma; LPO is expressed as nmol malonaldehyde formed/g Hb/min.

Table 2. Systemic antioxidant status of active non-segmental vitiligo (NSV) vs. active segmental vitiligo (SV) and stable NSV vs. stable SV in 16–25 yr of age group

	Parameters						
Type of vitiligo	SOD	CAT	GPx	G6PDH	GSH	LPO	Vit E
Active NSV (n = 13)	2566 ± 123.34	303.2 ± 2.96	12.78 ± 0.96	5.537 ± 0.16	25.63 ± 0.89	193.1 ± 18.78	8.682 ± 1.93
Active SV (n = 4)	2572 ± 140.90	307.1 ± 8.64	16.35 ± 2.05	5.525 ± 0.19	26.51 ± 2.40	190.9 ± 16.38	7.658 ± 1.86
Stable NSV (n = 8)	2598 ± 125.87	308.8 ± 3.47	15.26 ± 1.03	5.613 ± 0.28	25.87 ± 1.21	193.6 ± 23.30	8.617 ± 1.64
Stable SV (n = 4)	2514 ± 121.27	311.4 ± 6.18	12.88 ± 1.57	5.538 ± 0.26	26.24 ± 1.29	189.5 ± 24.29	8.450 ± 1.65

Values are expressed as mean ± SE.
SOD is expressed as U/g Hb; CAT is expressed as *k*/g Hb/s; GPx is expressed as μmol GSH utilized/g Hb/s; G6PDH is expressed as mmol NADP reduced to NADPH/g Hb/min; GSH is expressed as mg GSH/dl blood; Vitamin E is expressed in μg Vit E/ml of plasma; LPO is expressed as nmol malonaldehyde formed/g Hb/min.

hexose monophosphate shunt pathway is the only source of NADPH, which is necessary to protect the cell from oxidative damage (42, 43). Significantly lower GSH levels were observed in all age groups of vitiligo patients compared with controls (Fig. 5). Passi et al. (23) showed that epidermal GSH levels of active vitiligo patients were significantly lower compared with controls. Changes in plasma vitamin E levels in the present study were non-significant (Fig. 7) and our results are in concordance with Picardo et al. (14). Vitamin E is the only lipid-soluble antioxidant concentrated mainly in plasma membrane and erythrocyte membranes. Eighty percentage of vitamin E in serum is in the free form. It terminates free radical reactions acting as a chain breaking antioxidant. A significant increase in LPO levels was also observed in all age groups of vitiligo patients, where 36–45 yr age group showed a 55% increase compared with controls (Fig. 2). However, non-significant changes in plasma lipoperoxides of vitiligo patients were reported by Picardo et al. (14).

Thus increased levels of erythrocyte SOD (44, 45) in vitiliginous patients could enhance the systemic production of H_2O_2. The downstream antioxidant enzymes that neutralize H_2O_2 are CAT and GPx. Although CAT levels are unchanged, a significant decrease in GPx was observed in the patients. As GPx also neutralizes lipid hydroperoxides, low levels of GPx in vitiliginous patients could lead to oxidative stress, which is evident in high LPO levels of these patients. Plasma vitamin E levels of the vitiligo patients remain unchanged, however, with significantly lower GSH and NADPH levels (due to reduced levels of G6PDH), the non-enzymatic cycle may not proceed to completion. Hence the free radicals may accumulate and contribute to the build up of oxidative stress in the system. GSH is not only required for glutathione reductase but also acts as a substrate for GPx. Thus low levels of GPx, G6PDH and GSH could contribute to oxidative stress in the system as is evident by high LPO levels in vitiligo patients of our study.

In conclusion, impairment in the systemic antioxidant system is observed in this study (44, 45) indicating that melanocyte damage in vitiligo may be linked with generalized oxidative stress. Although increased oxidative stress is observed in the age group of 36–45 yr, this is not statistically significant among the groups. No significant differences are observed in the antioxidant status between segmental and non-segmental types of vitiligo. Also there is no significant difference found in active non-segmental vs. active segmental and stable non-segmental vs. stable segmental vitiligo. Thus, systemic oxidative stress may have a pathophysiological role in all the age groups and types of vitiligo. The present study is the first report on the age-dependent study of antioxidant parameters of vitiligo patients in Baroda, Gujarat state, India.

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