Study of oxidative stress in peripheral blood of Indian vitiligo patients

Sudha S. Deo, Ameya R. Bhagat, Rajnikant N. Shah[1]

Department of Immunology, Sir. H.N. Medical Research Society, and [1]Sir. H.N. Hospital and Research Centre, Girgaum, Mumbai, Maharashtra, India

ABSTRACT

Background: Vitiligo is an acquired skin disease that involves the interplay of complex genetic, immunological, neural and self-destructive mechanisms in its pathogenesis. According to autocytotoxic hypothesis, oxidative stress has been suggested to be the initial pathogenic event in melanocyte degeneration. **Objectives:** The aim of our investigation was to evaluate the role of oxidative stress by studying the role of catalase (CAT) in the destruction of melanocytes in patients with vitiligo and compare the same in healthy normal controls. **Materials and Methods:** We determined the serum catalase enzyme by ELISA method. The catalase activity was studied in two groups, Group I—localized vitiligo: (i) active stage, (ii) static or inactive stage and Group II—generalized vitiligo: (i) active stage, (ii) static or inactive stage patients, and the levels were compared with healthy controls. **Results:** Group I active stage patients showed significant difference in the catalase levels with a $P < 0.044$ when compared with healthy controls, whereas Group II static stage patients did not show any significant difference ($P < 0.095$) although the catalase activity was increased. **Conclusion:** Our study could not explain the cause of melanocyte damage in patients in the active stage of the disease. The increase in the oxidative stress as detected by catalase activity was more significant in Group I active disease than Group II active disease patients although the levels were higher than the healthy normals. This is the first study conducted on active and static stage of vitilgo in India. It is possible that the number of compounds of hydrogen peroxide produced is not balanced by the production of catalase in the body.

Key words: Enzyme linked immunoassay, melanocyte destruction, vitiligo catalase activity

Access this article online

Website: www.idoj.in

DOI: 10.4103/2229-5178.120637

Quick Response Code:

Address for correspondence:
Dr. Sudha S Deo, Sr. Scientist, Sir. H.N. Medical Research Society, Sir. H.N. Hospital and Research Centre, Girgaum, Mumbai - 400 004, Maharashtra, India.
E-mail: sudha_deo@hnhospital.com

INTRODUCTION

Despite much research, the etiology of vitiligo and the causes of melanocyte death are not clear. At least three pathogenic mechanisms—immunological, neural, and biochemical—have been suggested, but none can completely explain the cause of the disease.[1,2] One of the newer theories in the cause of vitiligo is oxidative stress. Oxidative stress is an overaccumulation of hydrogen peroxide (H_2O_2) in the skin. Every person develops H_2O_2 in the skin, as a result of natural biological processes. An enzyme called "catalase" normally breaks down the H_2O_2 in the skin into water and oxygen. However, people with vitiligo may have a problem manufacturing catalase in their body or delivering the catalase to the skin.[3-5] Many experts feel that the increase in H_2O_2 in the skin may be one of the root causes of vitiligo, and in fact, studies have suggested that most people with vitiligo exhibit this "oxidative stress."[6] Some have even theorized that the autoimmune response in vitiligo could be related to the oxidative stress.[7] Viruses and bacteria produce H_2O_2, and so the presence of it in our skin could conceivably be triggering the immune cells to attack our pigment cells. Many people report first noticing their vitiligo after traumatic events, periods of stress, or severe sunburns. Special machines used to measure levels of H_2O_2 in the skin show that these events may actually increase oxidative stress in the skin.

Some findings have suggested that oxidative stress may be an important phenomenon in the pathophysiology of the disease. Hydroxyl ions increase the pH in the epidermis, and as a consequence glutathione reductase activity is increased in patients with vitiligo compared with controls. Based on these new results, together with the previously reported calcium transport defect, a new hypothesis has been formulated for the pathogenesis of vitiligo.[8]

Catalase plays an important role in the conversion of H_2O_2 to water and oxygen. Absence of catalase

can lead to accumulation of H_2O_2, and according to the cytotoxic hypothesis, oxidative stress has been suggested to be the initial pathogenic event in melanocyte degeneration with H_2O_2 accumulation in the epidermis of patients with active disease.[9-11]

All these abnormal biochemical events in the epidermis of vitiligo patients may contribute to increased levels of H_2O_2. The presense of normal catalase activity helps in the breaking down of H_2O_2 but excess H_2O_2 can also deactivate the enzyme. Low catalase activities have previously been reported in the epidermis of vitiligo patients, whether this defect is due to an increase in H_2O_2 from the defective 6BH4 pathway or due to a separate problem in the catalase enzyme is unknown. What has been shown is that treatment with pseudocatalase, a bus manganese III-EDTA $(HCO_3)_2$ synthetic catalase substitute, has promoted repigmentation in vitiligo patients, along with restoring DH enzyme activity and a return to normal 7BH4 levels in the epidermis.[8]

Reduced antioxidant activity in peripheral blood mononuclear cells of vitiligo patients has also been observed, with increased superoxide dismutase activity, and reduced catalase, glutathione, and vitamin E levels. This variability in antioxidant levels was seen exclusively in subjects with active disease. These changes in antioxidants could be responsible for the generation of intracellular reactive oxygen species (ROS) in vitiligo patients and may be due in part to an observed mitochondrial impairment.

With the increase in vitiligo patients, we felt it appropriate to study the catalase activity in serum of vitiligo patients and compare the same in healthy normal individuals.

MATERIALS AND METHODS

The study involved human vitiligo patients attending the OPD of the skin department and healthy normal individuals taken as controls for comparison.

Patients

Clinically confirmed cases (*N* = 80) of Vitiligo were included in this study. These patients were selected by our very specialized dermatologist on the basis of clinical symptoms. The clinical history, family history, the environmental factors were all noted on the clinical proforma. Informed consent was taken from the patients for obtaining blood samples for the study of all the parameters under the project, and ethical approval was taken from the scientific advisory committee of the Sir. H.N. Hospital and Research Centre and the project was sanctioned by the Management of Medical Research Society.

These patients were broadly categorized into two groups, Group I and Group II, depending on the involvement of the disease and further subdivided according to the activity of the disease as active stage (if new lesions were seen in last 1 year) or inactive stage (if no lesions were seen during the full year).

(a) Group I—localized vitiligo
   (i) Active stage
   (ii) Static or inactive stage.
(b) Group II—generalized vitiligo
   (i) Active stage
   (ii) Static or inactive stage.

Controls

A control group of healthy normal individuals (N = 30), which were age and sex matched, were taken for comparison in this study. These individuals were free of any infection and previous illness and with no family history of vitiligo.

Peripheral blood collection

Venous blood (about 3 ml) was collected in plain vacutainers and incubated at room temperature (RT) for the serum to be separated. The serum separated by centrifugation was then stored at − 20°C to be used for ELISA assay for further analysis of catalase enzyme activity.

The method used was ELISA assay for catalase enzyme. Activity of catalase is then determined by the following formula as mentioned in the protocol.

Assay protocol for catalase enzyme by ELISA

100 μl of assay buffer (containing 2 ml of assay buffer concentrate containing 100 mM potassium phosphate of pH 7.0 is diluted in 18 ml of HPLC grade water) was added to all the wells. 30 μl of methanol, 20 μl of each standard diluted with sample buffer, and formaldehyde as given in the protocol in concentrations of 0, 5, 15, 30, 45, 60, and 75 were then added to the standard wells and 20 μl of CAT control were then added into the control wells. 20 μl of the serum sample diluted 1:2 with the sample buffer (5 ml concentrate of 25 mM potassium phosphate pH 7.5 containing 1 mM EDTA and 0.1% BSA is diluted in 45 ml) was then added to all the sample wells. The reaction was initiated by adding 20 μl of H_2O_2 to all the wells. Precise time of initiation was noted. The plate was covered with a plate cover and incubated on a shaker for 20 minutes at RT. To this were added 30 μl of H_2O_2 to terminate the reaction. And then 30 μl of Purpald was added as a chromogen. The plate was covered with a plate cover and incubated for 10 minutes at RT. On the shaker 10 μl of potassium periodate was added to each well. The plate was then covered with a plate cover and incubated for 5 minutes at RT on a shaker. The absorbance was then read at 540 nm using a plate reader.[12]

Determination of the reaction rate

The average absorbance of each standard and sample was calculated. The average absorbance of the standard A (Blank)

was subtracted from itself and all the standards and samples. The corrected absorbance of standards was then plotted against the final formaldehyde concentration (μM) for a typical standard curve. The formaldehyde concentration of the samples was calculated using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

Formaldehyde (μM) = Sample absorbance-*y* intercept × 0.17 ml slope 0.02 ml.

The catalase activity of the sample was calculated using the following equation.

One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.[13]

CAT activity = μM of sample × sample dilution = mmol/min/ml 20 min.

Statistical analysis

The statistical analysis was performed using SPSS version 16 software, and the data were expressed as mean ± SE. $P < 0.05$ was considered as statistically significant.

RESULTS

The study consisted of three groups, of which Group I contained 10 patients with 9 showing active condition while only 1 showed static condition with 4 males and 6 females. Group II involved/consisted of 70 patients with 49 of these showing the active state while 21 were static, and the male to female ratio being almost 1:1. The third group consisted of normal healthy controls with 13 males and 17 females with age range of 21-45 years. Most of the patients in Group II were of elderly age group [Table 1].

Table 2 illustrates the serum catalase activity across various groups. There was no significant catalase enzyme activity in any of the groups when compared with the control group, although Group II patients showed higher concentrations of catalase levels in the serum.

It is seen that Group I active stage patients showed significant difference in the catalase levels with a $P < 0.044$ when compared with controls in Table 3, whereas Group II static stage patients did not show any significant difference ($P < 0.095$) when compared with controls.

Table 1: The general characteristics of the study group

Groups	No. of subjects (*N*)	Stage		Sex ratio male:female	Age range (years)
		Active	Static		
I	10	9	1	4:6	5-52
II	70	49	21	30:40	5-72
Control	30	-		13:17	21-45

Table 2: Comparison of catalase enzyme activity in Group I, Group II and controls

Groups	Mean±SE (nmol/min/ml)	*P* value
I (*N*=10)	36.95±11.25	0.274
II (*N*=70)	35.05±3.34	0.095
Controls (*N*=30)	28.69±4.98	-

Group I active *N*=9 & not 10 hence mean±SE changed & control *N*=30

Table 3: Catalase activity in different stages of the disease

Groups	Active stage	
	Mean±SE (ng/ml)	*P* value
I (*N*=9)	26.34±4.17	0.044*
II (*N*=49)	35.05±4.00	0.095
Controls (*N*=30)	28.69±4.98	-
	Static stage	
I (*N*=9)	-	-
II (*N*=21)	35.95±7.42	0.274
Controls (*N*=30)	28.69±4.98	-

DISCUSSION

One of the hypotheses for vitiligo is the self destruct theory that suggests the role of oxidative stress. Oxidative stress has been considered as the initial pathogenic event in melanocyte destruction.[14,15] Free radicals such as superoxide, H_2O_2, and nitric oxide are molecules that occur during several physiological and pathological processes. These free radicals are scavenged continuously by antioxidant enzymes such as catalase. In oxidative stress, there is insufficient antioxidant activity leading to excessive accumulation of free radicals that damage cellular compounds such as proteins, carbohydrate, DNA, and lipid.

Studies on vitiligo by Arican *et al*.[16] showed that the erythrocyte catalase activity from the blood of active localized vitilgo patients was significantly lower than that of controls, but Hazneci *et al*.[10] did not find any statistical difference.

Studies conducted in our laboratory showed that serum CAT activity was not significantly different in Group I and Group II patients when compared with controls. Group II patients of both the static and active groups showed increased CAT activity when compared with healthy controls, but only the static group showed significant catalase activity. Group I patients in the active group did show low CAT activity compared with Group II patients, though not statistically significant. Our hypothesis is that catalase is not delivered of adequately to the skin in vitiligo.

Schallreuter *et al*.[11] obtained epidermal suction blisters from involved and uninvolved skin of vitiligo patients and healthy controls and showed consistent reduction in CAT activity and demonstrated H_2O_2 accumulation in the skin.

D'Anna *et al*.[17] studied the effect of mitochondrial impairment in the active stage of the disease and observed low CAT activity in polymorphs but not in erythrocytes. High levels of superoxide in erythrocyte and nitrate levels in plasma were seen.

Yildirium *et al*.[7] showed that there is an imbalance in oxidant and antioxidant system in vitiligo patients.

In 2002, case–control and family-based association studies for the catalase gene (*CAT*) in vitiligo patients were conducted. The *CAT* gene was selected as a candidate gene because of the reduced catalase enzyme activity and accompanying accumulation of excess H_2O_2 in the entire epidermis of vitiligo patients. One of three *CAT* genetic markers studied was found to be informative in the genotypic analysis of Caucasian vitiligo patients and control subjects. Both case–control and family-based genetic association studies of this genetic marker suggested a possible association between the *CAT* gene and vitiligo susceptibility. The observations that individuals who carried different gene variations (i.e. were heterozygous) had vitiligo more frequently than controls and that the one variant is transmitted more frequently to patients than controls suggest that linked mutations in or near the CAT gene might contribute to a quantitative deficiency of catalase activity in the epidermis and the accumulation of excess H_2O_2.[18,19]

ACKNOWLEDGMENTS

The authors wish to thank the Director and the Management of the Sir. H.N. Medical Research Society for providing the funds to conduct this project.

REFERENCES

1. Handa S, Kaur I. Vitiligo: Clinical findings in 1436 patients. J Dermatol 1999;26:653-7.
2. Dell'anna MI, Urbanelli S, Mastrofrancesco A, Camera E, Iacovelli P, Leone G, *et al*. Alterations of mitochondria in peripheral blood mononuclear cells of vitiligo patients. Pigment Cell Res 2003;16:553-9.
3. Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci 2004;61:192-208.
4. David S, Catalase G. The PDB newsletter. RCSB Protein Data Bank. 2004.
5. Boon EM, Downs A, Marcey D. "Catalase: H_2O_2: H_2O_2 Oxidoreductase". Catalase Structural Tutorial Text. Available from: http://biology.kenyon.edu/BMB/Chime/catalase/frames/cattx.htm [Last accessed on 2007 Feb 11].
6. Gaetani G, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant role of catalase in the disposal of hydrogen peoxidase within human erythrocytes. Blood 1996;87:1595-9.
7. Yildirim M, Baysal V, Inaloz HS, Kesici D, Delibas N. The role of oxidants and antioxidants in generalized vitiligo. J Dermatol 2003;30:104-8.
8. Schallreuter KU, Moore J, Wood JM, Beazley WD, Peters EM, Marles LK, *et al*. Epidermal H_2O_2 accumulation alters tetrahyrobiopterin (6BH4) recycling in vitiligo: Identification of general mechanism in regulation of all 6BH4 dependent processes? J Invest Dermatol 2001;116:167-74.
9. Yildrim M, Baysal V, Inaloz HS, Can M. The role of oxidants and anti-oxidants in generalized vitiligo at the tissue level. J Eur Acad Dermatol Venereol 2004;18:683-6.
10. Hazneci E, Karabulut AB, Ozturk C, Batçioglu K, Dogan G, Karaca S, *et al*. A comparative study of superoxide dismutase, catalase and glutathione peroxidase activities and nitrate levels in vitiligo patients. Int J Dermatol 2005;44:636-40.
11. Schaullreuter KU, Wood J, Berger J. Low catalase levels in the epidermis of patients with vitiligo. J Invest Dermatol 1991;97:1081-5.
12. Johansson LH, Borg LA. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal Biochem 1988;174:331-6.
13. Wheeler CR, Salzman JA, Elsayed NM, Omaye ST, Korte DW Jr. Automated assays for superoxide dismutase, catalase, glutathione peoxidase, and glutathione reductase activity. Anal Biochem 1990;184:193-9.
14. Jain A, Mal J, Mehndiratta V, Chander R, Patra SK. Study of Oxidative Stress in Vitiligo. Indian J Clin Biochem 2011;26:78-81.
15. Gopal KV, Rao GR, Kumar HK, Rao MV, Vasudev P. Vitiligo: A part of systemic autoimmune process. Indian J Dermatol Venerol 2007;73:162-5.
16. Arican O, Kurutus EB. Oxidative stress in the blood of patients with active localised vitiligo. Acta Dermatovenerol Alp Panonica Adriat 2008;17:12-6.
17. Dell›Anna ML, Maresca V, Briganti S, Camera E, Falchi M, Picardo M. Mitochondrial impairment in peripheral blood mononuclear cells during the active phase of vitiligo. J Invest Dermatol 2001;117:908-13.
18. Casp CB, She JX, McCormack WT. Genetic association of the catalase gene (CAT) with vitiligo susceptibility. Pigment Cell Res 2002;15:62-6.
19. Agrawal D, Shajil EM, Marfatia YS, Begum R. Study on the antioxidant status of vitiligo patients of different age groups in Baroda. Pigment Cell Res 2004;17:289-94.

Cite this article as: Deo SS, Bhagat AR, Shah RN. Study of oxidative stress in peripheral blood of Indian vitiligo patients. Indian Dermatol Online J 2013;4:279-82.

Source of Support: Nil, **Conflict of Interest:** None declared.