CHAPTER III A

THE ANTIOXIDANT STATUS OF VITILIGO PATIENTS OF DIFFERENT AGE GROUPS

3A.1. Introduction

Vitiligo is an idiopathic, acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches of different size and shape and affects 1-2% of the world population (Moscher et al 1999; Nordlund and Ortonne 1998). Based on a few dermatological outpatient records, the incidence of vitiligo is found to be 0.5-2.5% in India (Handa and Kaur 1999; Das et al 1985). Gujarat and Rajasthan states have the highest prevalence i.e. ~8.8% (Valia and Dutta 1996). Vitiligo is classified into vulgaris, focal, acrofacial, segmental and universal types. Vulgaris is the type of vitiligo in which the lesions are found in typical zones with symmetrical distribution. In focal vitiligo one or more patches are found in one area but not in segmental pattern. Acrofacial vitiligo affects face and distal extremities; whereas in segmental vitiligo one or more macules are found in dermatomal unilateral distribution. In universal vitiligo the depigmentation involves more than 80% of the body (Hann and Nordlund 2000). The active and stable phases of vitiligo are defined on the basis of the progression or appearance of lesions in the last three months (Dell'Anna et al 2001). Though vitiligo is extensively addressed in the past five decades, its etiology is still being debated (Taieb 2000; Le Poole et al 1993; Ortonne and Bose 1993; Cucchi et al 2003; Ongenae et lal 2003; Boisseau-Garsuad et al 2002). Several hypotheses have been proposed to explain the pathogenesis of vitiligo and oxidative stress hypothesis considers a systemic involvement in the course of the disease (Picardo et al 1994; Yildirim et al 2003). Epidermal or systemic oxidative stress could act as the initial triggering event in melanocyte degeneration (Maresca et al 1997). In accordance with this hypothesis, the present study was undertaken and Vadodara was selected for this study, as it is one of the highly industrialized cities in Gujarat.

We have analyzed the blood/systemic enzymatic antioxidant status such as catalase, superoxide dismutase, glutathione peroxidase, glutathione *S* transferase and glutathione reductase; non-enzymatic antioxidant molecules such as reduced

glutathione and vitamin E; erythrocyte lipid peroxidation levels and glucose 6 phosphate dehydrogenase activity in different age groups as well as different clinical types (Chapter 3 B) of Gujarat vitiliginous patients and age matched controls.

3A.2. Materials and methods

Chemicals

Drabkin's reagent and cyanmethemoglobin standard were procured from Monozyme, Secunderabad. Thiobarbituric acid (TBA), tetra methoxy propene (TMP) and reduced glutathione (GSH) were procured from Sigma Chemical Co., U.S.A. NBT, PMS, NADH, DTNB, NADP, CDNB and oxidized glutathione were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals are of Analytical Reagent (AR) grade and procured from Qualigens Fine Chemicals, Mumbai, India.

Patients and controls

Information on general clinical history and other details of the patients volunteered were obtained with the help of vitiligo questionnaire (Annexure I). Blood was collected from the vitiligo patients visiting Sir Sayajirao Gaikwad Medical College hospital and Param Ayurvedic hospital, Sakarda who were not suffering from any other disease after a written consent was obtained from them. Also blood was collected from one twenty six age matched healthy controls. For the estimation of antioxidant parameters in the blood, 124 vitiligo patients were divided into different age groups (5-15 yrs, 16-25 yrs, 26-35 yrs 36-45 and 46-55 yrs) and different clinical types.

Blood collection

Five ml of blood was collected from each patient in EDTA coated bulb and carried to the department on ice. Reduced glutathione levels were estimated from the fresh whole blood immediately. Other parameters of oxidative stress were

estimated in hemolysate except glutathione *S* transferase and vitamin E, which were estimated in plasma.

Sample preparation

Whole blood was centrifuged at 3000 rpm for 10 minutes. Plasma was separated and stored in deep freezer (-20^{0}C) until use. Erythrocyte sediment was washed thrice with PBS and hemolysate was prepared by adding distilled water corresponding to the amount of plasma separated. The hemoglobin concentration of hemolysate was done by Drabkin's method.

Estimation of blood antioxidant parameters

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

Hemoglobin estimation: Hemoglobin estimation was done by cyanmethemoglobin method (Dacie and Lewis 1968).

Principle

Drabkin's reagent (ferricyanide) converts the hemoglobin to cyanmethemoglobin (CMG) and the absorbance of CMG is proportional to the hemoglobin concentration. The optical density was measured at 540 nm against distilled water.

Reagents
Drabkin's reagent
Cyanmethemoglobin standard (65mg/dl)

Protocol

Reagent	**Blank**	**Control**	**Test**
CMG standard	-	3 ml	-
Sample	-	-	20 µl
Drabkin's reagent	5 ml	-	5 ml

Mixed well and kept for 5 minutes. Read the absorbance of the test and CMG standard against distilled water at 540 nm separately.

Calculation

$$\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times \frac{251}{1000} \times 65$$

251 is the dilution factor

1000 is to convert mg/dl to gm/dl

Unit: gHb/ dl (gram hemoglobin/100 ml)

Superoxide Dismutase (SOD) estimation

Erythrocyte SOD was assayed according to the method of Nishikimi et al (1972).

Principle: Mixtures of NADH and phenazine methosulfate (PMS) generate superoxide under non – acidic conditions via the univalent oxidation of reduced PMS. NBT serves as a detector molecule for superoxide through reduction in to a stable, blue colored formazone product, which can be measured at 560nm.

Reagents	
PBS (pH 7.4)	0.1M
Sodium pyrophosphate (pH 8.3)	0.052 M
PMS	186 µM
NBT	300 µM
NADH	780 µM
Glacial acetic acid	
Butanol	

Sample preparation

Erythrocyte sediment was washed thrice with PBS. Hemolysate was prepared with hemoglobin concentration of about 1g Hb/dl.

Protocol

Reagents	Control	Test
Sodium pyrophosphate buffer	1.2 ml	1.2 ml
PMS	0.1 ml	0.1 ml
NBT	0.3 ml	0.3 ml
Hemolysate (1g Hb/dl)	----	0.01 ml
Distilled water	1.21 ml	1.2 ml
NADH	0.2 ml	0.2 ml

All the tubes were incubated for 90 seconds at 37^{0}C, reaction was terminated by adding 1 ml glacial acetic acid and shaked vigorously. Reduced NBT was extracted in 4 ml butanol. Tubes were centrifuged and absorbance was read at 560 nm against butanol blank.

Calculation

$$\text{SOD (U/g Hb)} = \frac{\text{OD}^{\text{control}} - \text{OD}^{\text{test}}}{\text{½ of OD}^{\text{control}}} \times \frac{100}{0.01} \times \frac{60}{90} \times \frac{1}{\text{Hb Con (g/dl)}}$$

Unit: One unit of SOD is defined as the amount of enzyme required to inhibit NBT reduction by 50% as compared to control.

Catalase (CAT) estimation

Catalase activity in the hemolysate was assayed by the method of Aebi (1984).

Principle: Catalase is a heme-containing enzyme, which catalyzes dismutation of hydrogen peroxide into water and oxygen. Decomposition of hydrogen peroxide by catalase is measured spectrophotometrically at 240 nm, since hydrogen peroxide absorbs UV light maximally at this wavelength.

Reagents	
Phosphate buffer (pH 7.0)	50 Mm
Hydrogen peroxide	8mM
PBS (pH 7.4)	0.1 M

Sample preparation

Hemolysate was prepared and Hb concentration was adjusted to 5g/dl. Just before assay, 0.02 ml hemolysate was diluted up to 10 ml with phosphate buffer and this diluted solution was used for enzyme analysis.

Protocol

Reagents	Blank	Test
Sample	2.ml	2 ml
Phosphate buffer	1.0ml	-
H_2O_2	-	1.0ml

Immediately after adding H_2O_2 decrease in the absorbance was recorded at every 5 second interval for 15 seconds at 240nm.

Calculation

$$\text{CAT activity} = \frac{2.303}{\Delta t} \times \log \frac{E1}{E2} \times \text{Dilution factor}$$

Unit – k/gHb OR mmoles of H_2O_2 decomposed / g Hb/ sec

Glutathione Peroxidase (GPX)

Erythrocyte glutathione peroxidase was assayed according to the method of Paglia and Valentine (1967). Glutathione peroxidase catalyzes the reduction of various organic hydroperoxides as well as hydrogen peroxide with glutathione as hydrogen donor.

Principle

Glutathione peroxidase catalyzes the oxidation of GSH to GSSG by hydrogen peroxide.

$$2\,GSH + ROOH \xrightarrow{GPx} GSSG + H_2O + ROH$$

ROOH is lipid peroxide and the utilization of GSH by the enzyme is measured by means of the colored substance produced by the reaction with DTNB at 412 nm.

Reagents	
Phosphate buffer (pH 7.0)	0.4 M
Precipitating reagent	Glacial metaphosphoric acid 1.67 g, EDTA 0.20 g, NaCl 30 g and total volume was made up to 100 ml with distilled water.
GSH	2mM
Potassium cyanide	15 mM
Hydrogen peroxide	10 mM
Na_2HPO_4	0.4 M
DTNB	40 mg DTNB in 100 ml 1% sodium citrate

Protocol

Hemolysate was prepared as mentioned in earlier methods. The hemoglobin concentration of the hemolysate was adjusted to 0.25gHb/dl.

Reagents	Blank	Control	Test
Buffer	0.1 ml	0.1 ml	0.1 ml
GSH	---	0.1 ml	0.1 ml
KCN	0.1 ml	0.1 ml	0.1 ml
Hemolysate	0.01 ml	----	0.01 ml
Distilled water	0.19 ml	0.1 ml	0.09 ml

Incubated at 37^{0}C for 5 minutes, added 0.1 ml of H_2O_2 and again incubated for 3 minutes, added 0.4 ml precipitating reagent, centrifuged at 3000 rpm for 10 minutes. Took 0.6 ml of the supernatant and added 0.6 ml Na_2HPO_4 and 0.03 ml of DTNB and measured the absorbance at 412 nm.

Calculation - Calculation was done according to the slope calculated from the standard graph of GSH as given below.

$$\frac{OD^{control} - OD^{test}}{Slope} \times \frac{1}{3} \times \frac{100}{0.01} \times \frac{1}{Hb\ Con\ (gHb/dl)}$$

Unit: µmoles of GSH utilized / gHb/sec

Reduced Glutathione (GSH)

Total reduced glutathione in the whole blood was determined by the method of Beutler et al (1963).

Principle: Red cell contains GSH as a non–protein sulfhydryl compound. 5-5′ Dithiobis (2–nitrobenzoic) acid (DTNB) is a disulfide compound, which is readily reduced by sulfhydryl compounds forming a highly colored yellow anion, which was read at 412 nm.

Reagents

PBS (pH 7.4)	0.1M
Precipitating Reagent and DTNB	Prepared similarly as that of GPX assay
Na_2HPO_4	0.3M
Standard GSH solution	2 mM (Standard range 10-100 µg)

Fresh whole blood was used as the sample

Protocol

Reagents	**Blank**	**Test**
Sample	-	0.1ml
Distilled water	1.0ml	0.9ml
Precipitating reagent	1.5ml	1.5ml
Kept tubes for 5 minutes and centrifuged 3000 rpm for 15 minutes		
Supernatant	0.5ml	0.5ml
Na_2HPO_4 solution	2.0ml	2.0ml
DTNB	0.25ml	0.25ml

Calculation - Calculation was done according to the slope calculated from the standard graph.

Unit – GSH mg % (blood)

Vitamin E

The plasma vitamin E level was estimated by the method of Hansen and Warwick (1969).

Principle: Eighty percent of the vitamin E in the plasma is in the free form. Vitamin E is extracted into hexane and then quantitated by measuring the fluorescence emission at 325 nm with an excitation wavelength of 295 nm.

Reagents	
Methanol	HPLC grade
Hexane	HPLC grade
Vitamin E Standard	Standard range 2µg - 20 µg

Sample preparation: After the centrifugation of the blood at 3000 rpm for 10 minutes, plasma was separated.

Protocol

Following reagents were added in 15 ml stoppered glass tubes. All the glassware used for the vitamin estimation was washed thoroughly with 8 M nitric acid followed by double distilled water wash.

Reagents	**Blank**	**Test**
Plasma	---	0.5 ml
Double distilled water	1.2 ml	0.7 ml
Methanol	2 ml	2 ml
Hexane	5 ml	5 ml

After the addition of methanol vortexed for 30 seconds and after adding hexane vortexed for 1 minute. Centrifuged at 3000 rpm for 15 minutes.

Calculation: - Calculation was done according to the slope calculated from the standard graph.

Unit: µg vitamin E/ml plasma

Glucose 6 Phosphate Dehydrogenase (G6PDH)

Erythrocyte Glucose 6 phosphate dehydrogenase was assayed by the method of Kornberg and Horecker (1955).

Principle: Glucose 6-phosphate dehydrogenase was assayed by measuring increase in the absorbance at 340 nm, which occurs due to the reduction of NADP

to NADPH when two electrons were transferred from glucose 6 phosphate to NADP in the reaction catalyzed by glucose 6 phosphate dehydrogenase.

$$\text{Glucose 6 P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{6 - phosphogluconolactone} + \text{NADPH} + \text{H}^{+}$$

Reagents	
Tris HCl Buffer (pH 7.6)	50 mM
NADP	2mM
Glucose 6 phosphate	6mM
$MgCl_2$	0.01 M
Triton X 100	10%

Sample preparation

Hemolysate was prepared as mentioned earlier. The Hb concentration of the hemolysate was adjusted to 1g Hb/dl.

Protocol

Reagents	**Blank**	**Test**
Tris HCl	0.1 ml	0.1 ml
Hemolysate	---	0.1 ml
Triton X 100	0.015 ml	0.015 ml
Distilled water	1.885 ml	1.785 ml
NADP	0.3 ml	0.3 ml
$MgCl_2$	0.1 ml	0.1 ml
$G6PO_4$	0.6 ml	0.6 ml

Immediately after adding $G6PO_4$ increase in the absorbance was recorded at every 5 seconds interval for 3 minutes at 340nm.

Calculation

$$\frac{\Delta \text{ OD}}{6.27 \times \text{Hb Con}} \times \frac{100}{0.1} \times \frac{1}{\text{Hb Con (gHb/dl)}}$$

6.25 is the molar extinction coefficient of NADP

Unit: mmoles of NADP reduced to NADPH/min/g Hb

Glutathione *S* Transferase (GST)

Plasma Glutathione S transferase was assayed by the method of Habig et al (1974).

Principle: 1 Chloro 2,4 dinitro benzene (CDNB) conjugates with the –SH group of reduced glutathione (GSH) and forms G-SDNB. The rate of formation of G-SDNB is measured spectrophotometrically at 340 nm for 3 minutes.

Reagents

Phosphate buffer (pH 6.5)	0.1M
GSH	100 mM
CDNB	100 mM, prepared in 95% ethanol

Protocol

Reagents	Blank	Test
Buffer	0.98 ml	0.93 ml
CDNB	0.01 ml	0.01 ml
GSH	0.01 ml	0.01 ml
Plasma	---	0.05 ml

After adding the first 3 reagents the tubes were incubated for 10 minutes. Then 0.05 ml of plasma was added and mixed well. The increase in the absorbance was recorded for 3 minutes at 340 nm.

Calculation

$$\frac{\Delta OD}{9.6} \times \frac{1}{0.05}$$

9.6 is the molar extinction coefficient of CDNB (9.6 L $mmol^{-1}cm^{-1}$)

Unit: mmoles of CDNB conjugated/ ml of plasma

Glutathione Reductase (GR)

Erythrocyte Glutathione reductase was assayed by the method of Smith et al (1988). Glutathione reductase is a ubiquitous enzyme required for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) concomitantly

oxidizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) to NADP.

Principle: The assay is based on the reduction of glutathione by NAPH in the presence of glutathione reductase.

$$NADPH + H^+ + GSSG \longrightarrow NADP + 2\,GSH$$

The reduced glutathione formed from the above reaction can then spontaneously reduce DTNB to TNB, which is yellow in color. The rate of increase in the absorbance was measured for 3 minutes at 412 nm.

Reagents

Phosphate buffer (pH 7.5)	0.2 M
DTNB	3 mM in 0.01 M phosphate buffer pH 7.5
NADPH	10 mM in 10 mM Tris HCl buffer pH 7.0
GSSG	20 mM

Sample preparation

Hemolysate was prepared as described earlier. Hemoglobin concentration of the lysate was adjusted to 1 g/dl which was used as the sample.

Protocol

Reagents	Blank	Test
Buffer	0.5 ml	0.5 ml
DTNB	0.2 ml	0.25 ml
Distilled water	0.195 ml	0.190 ml
NADPH	0.05 ml	0.05 ml
Hemolysate	----	0.005 ml
GSSG	0.05 ml	0.05 ml

The reaction was initiated by adding 0.05 ml of GSSG and the increase in absorbance was measured for 3 minutes at 412 nm.

Calculation

$$\frac{\Delta OD}{9.3} \times \frac{1}{0.05} \times \frac{100}{\text{Hb Con of the lysate}}$$

Lipid Peroxidation (LPO) levels

Erythrocyte lipid peroxidation was estimated according to the procedure of Beuge and Aust (1978).

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS) which gives a characteristic pink color that can be measured colorimetrically at 532 nm.

Reagents:

PBS (pH 7.4)	0.1 M
TBA Reagent	TBA 100mg, EDTA 46 mg, 20% TCA 5ml, 2.5 N HCl 5ml and total volume was made up to 20 ml with distilled water
Tetra Methoxy Propene (TMP)	10mM, as standard
Drabkin's reagent	

Sample preparation:

RBC pellet was prepared after centrifugation of the whole blood at 3000 rpm for 10 minutes. RBC pellet was washed thrice with PBS. 40µl of RBC pellet was added to 960µl of distilled water and shaked well. Hemoglobin content of the above solution was done by Drabkin's method.

Protocol

Reagents	**Blank**	**Test**
Sample	-	1.0ml
Distilled water	1.0ml	-
TBA reagent	1.0ml	1.0ml

The tubes were kept in boiling water bath for 20min, cooled under running tap water, centrifuged at 3000 rpm for 15 minutes and read the absorbance of the supernatant at 532nm.

Calculation: Calculation was done according to the slope calculated from the standard graph of TMP.

$$\frac{\text{OD of the Test}}{\text{Slope}} \times \frac{100}{\text{gHb}}$$

Units – nmoles of MDA formed/ g Hb

Statistical analysis

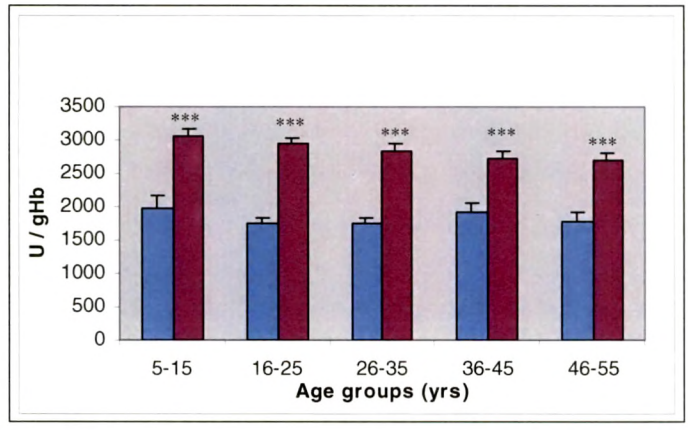
Results of vitiligo patients and age-matched controls were compared using the paired student's t test. One way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between different age groups and different clinical types of vitiligo patients utilizing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

3A.3. Results

This study was attempted to find whether age has any relevance for the onset of the disease. Vitiligo patients were divided into five age groups i.e. 5 – 15 yrs, 16 – 25 yrs, 26 – 35 yrs, 36 – 45 yrs and 46 - 55 yrs for the analysis of the antioxidant status. The age groups 5 – 15 yrs, 16 – 25 yrs, 26 – 35 yrs 36 – 45 yrs and 46-55 showed an increase in the erythrocyte SOD activity of 54.30% ($p<0.0001$), 66.93% ($p<0.0001$), 61% ($p<0.0001$), 42.18% ($p<0.0003$) and 51.32% ($p<0.0001$) respectively compared to the age matched controls (Figure 1). Overall patients of all age groups of vitiligo showed a significant increase in SOD activity compared to the controls (58.52%, $p<0.0001$). An increase in the LPO levels of the vitiliginous patients was observed i.e. 15.30 % ($p<0.017$), 10.74% ($p<0.006$), 17.9% ($p<0.0008$), 18.97% ($p<0.0023$) and 29.77% ($p<0.0015$) in the respective age groups compared

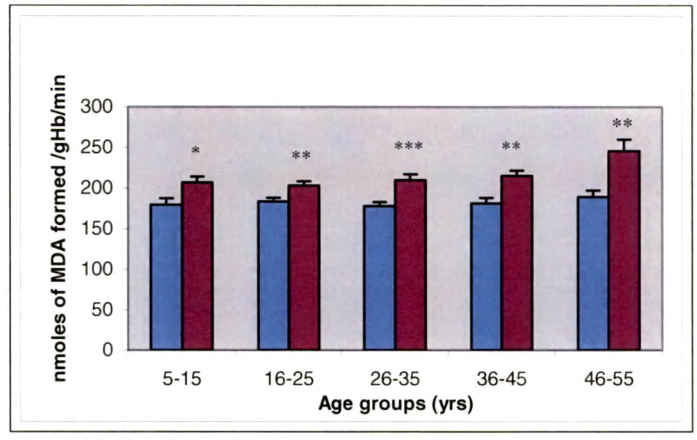
to their controls (Figure 2). Overall vitiligo patients (9.76%, $p<0.0001$) of all age groups showed a significant increase in LPO levels compared to controls.

Figure 1. Superoxide dismutase activity in erythrocytes of controls and vitiligo patients#



Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. ***, $p<0.001$

Figure 2. Erythrocyte lipid peroxidation levels in controls and vitiligo patients#

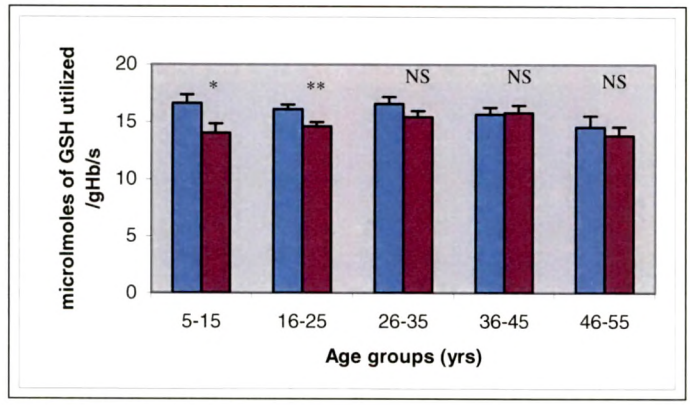


Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$

A significant decrease in GPX activity i.e. 15.42% ($p<0.026$) and 9.3% ($p<0.0073$) was observed in the 5-15 and 16-25 yrs age groups compared to their controls (Figure 3). Other age groups 26-35, 36-45 and 46-55 yrs did not show any significant change in the GPX activity compared to their respective controls. However overall patients showed a significant decrease in GPX activity compared to controls (11.17%, $p<0.0001$). A significant decline in G 6 PDH activity was also observed i.e. 49.03% ($p<0.0001$), 49.38% ($p<0.0001$), 43.42% ($p<0.0004$), 49.44% ($p<0.0001$) and 36.35% ($p<0.047$) in the respective age groups (Figure 4). Overall patients also showed significant decrease in G6PDH activity compared to controls (46.75%, ($p<0.0001$). Whole blood GSH was found to be decreased i.e. 17.47% ($p<0.0013$) in 36-45 yrs, and 21.98% ($p<0.035$) in 46-55 yrs age groups (Figure 5). Other age groups i.e. 5-15, 16-25 and 26-35 yrs did not show a significant change

in the GSH activity however, overall vitiligo patients showed a significant decrease in GSH levels (8.47%, $p<0.0014$) compared to controls.

Figure 3. Glutathione peroxidase activity in erythrocytes of controls and vitiligo patients#



#. Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. *, $p<0.05$; **, $p<0.01$ and NS, non significant

Figure 4. Glucose 6 phosphate dehydrogenase activity in erythrocytes of controls and vitiligo patients#

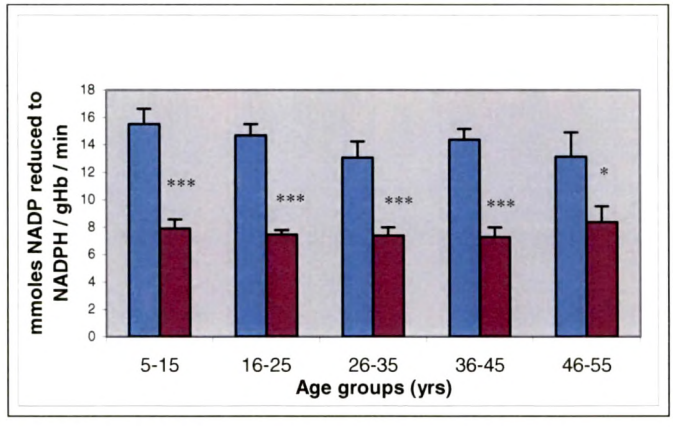


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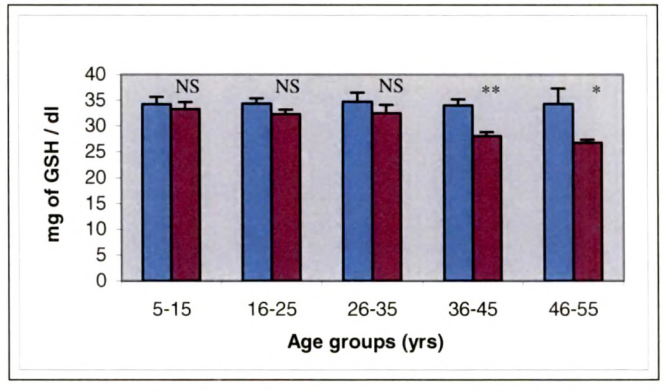
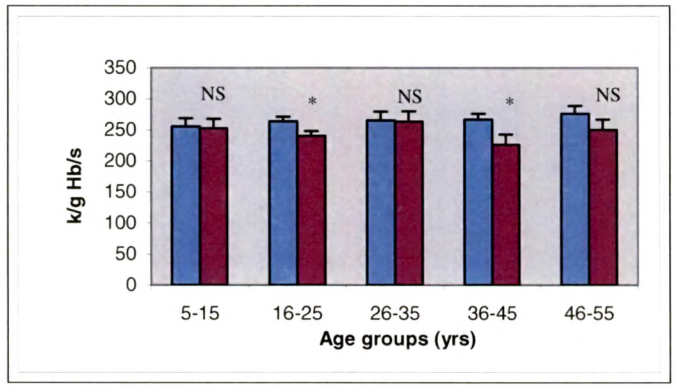
Figure 5. Whole blood GSH levels of controls and vitiligo patients#



Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. *, $p<0.05$; **, $p<0.01$, NS, non significant.

Erythrocyte catalase showed significant decrease in overall vitiligo patients (7%, $p<0.01$) as well as in 16-25 (9.13%, $p<0.03$) and 36-45 yrs (15.19%, $p<0.025$) age groups (Figure 6). However, plasma vitamin E levels showed no significant change in the vitiliginous patients compared to their controls (Figure 7). GST and GR activities also did not show any significant change in overall vitiligo patients as well as in any of the age groups (Figure 8 & Figure 9).

Figure 6. Catalase activity in erythrocytes of control and vitiligo patients[#]



[#]. Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. *, $p<0.05$; and NS, non significant

Figure 7. Vitamin E levels in the plasma of controls and vitiligo patients#

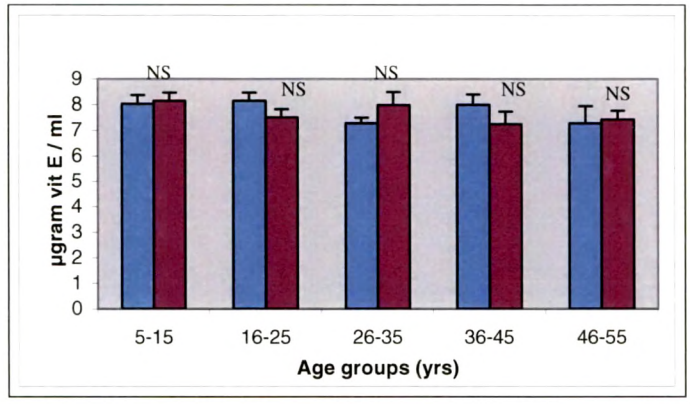


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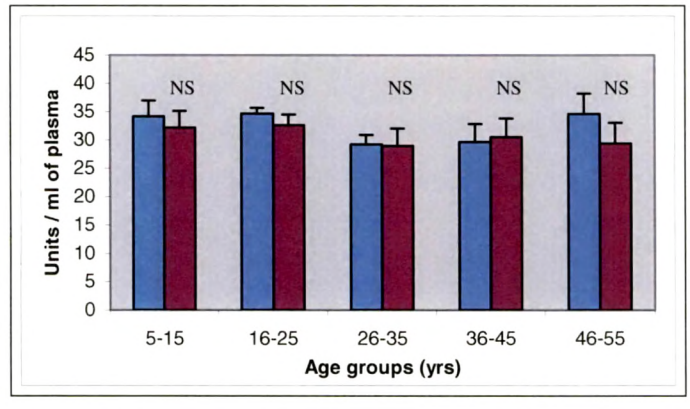
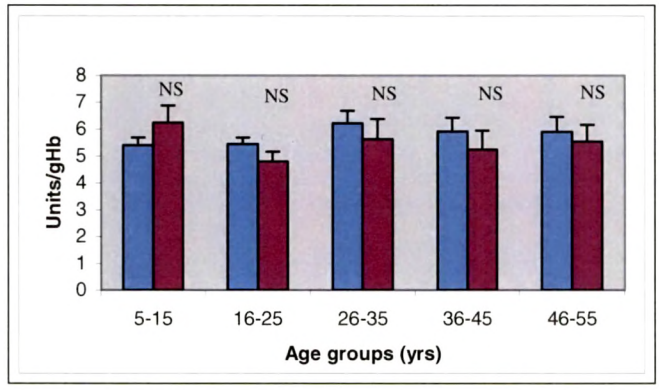
Figure 8. Glutathione *S* transferase activity in the plasma of controls and vitiligo patients#.



Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. NS, non significant

Figure 9. Glutathione reductase activity in the plasma of controls and vitiligo patients#



#. Values are given as mean ± SE of 19 and 22 individual observations in controls and patients in 5-15 age group; 45 and 57 individual observations in controls and patients in 16-25 age group; 24 and 18 individual observations in controls and patients 26-35 age group and 25 and 15 individual observations in controls and patients in 36-45 age group; 13 and 12 individual observations in controls and patients in 46-55 age group respectively. Blue bars are of controls and pink bars are of vitiligo patients. NS, non significant

Further, our analysis on ANOVA suggests that vitiligo might develop at any age and all age groups are equally susceptible to this disease. The age group 16-25 years that has more number of patients (n= 57) compared to other age groups was subdivided into segmental and non-segmental types (Table 1); and active and stable types of vitiligo (Table 2) and further analyzed for their antioxidant status. Interestingly, no significant difference was observed in the antioxidant status of the patients of segmental versus non-segmental as well as active versus stable vitiligo.

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

	SOD	CAT	GPX	GST	GR	G 6 PDH	GSH	VIT E	LPO
NSV n=52	2970 ± 82.99	239.9 ± 8.75	7.409 ± 0.39	31.42 ± 1.99	4.687 ± 0.34	7.409 ± 0.39	32.64 ± 0.98	7.595 ± 0.34	202.6 ± 5.47
SV n= 5	2655 ± 145.60	239.5 ± 25.86	7.652 ± 1.04	40.41 ± 6.67	5.556± 1.916	7.652 ± 1.04	28.45 ± 1.56	6.566 ± 1.10	207.6 ± 8.68

NS, Non segmental vitiligo, SV, Segmental vitiligo

GSH expressed as mg/dl of blood; CAT expressed as K/g Hb; SOD, GPX, G 6 PDH and GR expressed as U/g Hb; GST expressed as U/ ml of plasma; LPO expressed as n moles of malondialdehyde formed/gHb/min, Vitamin E expressed in µg/ml of plasma.

Table 2: Systemic antioxidant status of active vs. stable vitiligo patients in 16-25 years of age group

	SOD	CAT	GPX	GST	GR	G 6 PDH	GSH	VIT E	LPO
Active n=19	2737 ± 113.20	260.1 ± 11.56	14.37 ± 0.55	35.71 ± 4.07	5.219 ± 0.67	7.073 ± 0.60	31.23 ± 1.47	7.053 ± 0.37	201.7 ± 9.87
Stable n= 38	3016 ± 105.70	230.1 ± 11.10	14.72 ± 0.52	28.51 ±1.58	5.200 ± 0.45	7.358 ± 0.47	34.77 ± 1.30	7.451 ± 0.34	205.6 ± 6.02

Values are expressed as mean± SEM.

GSH expressed as mg/dl of blood; CAT expressed as K/g Hb; SOD, GPX, G 6 PDH and GR expressed as U/g Hb; GST expressed as U/ ml of plasma; LPO expressed as n moles of malondialdehyde formed/gHb/min, Vitamin E expressed in µg/ml of plasma

3A.4. Discussion

Oxidative stress acting as the triggering event in melanocyte degeneration in the etiopathogenesis of vitiligo is well established (Picardo et al 1994, Passi et al 1998). Oxidative stress hypothesis is based on the assumption that during melanin biosynthesis intermediates such as 3,4 - dihydroxy phenylalanine (DOPA), dopachrome and 5,6-dihydroxyindole (DHI) are formed that are known to be toxic to melanocytes (Hann et al 2000). Defective calcium homeostasis is also observed in vitiligo patients, which in turn affects the redox status of 6-biopterin/tetrahydrobiopterin equilibrium and monoamine oxidase activity among others (Schallreuter et al 1994; 1996a; 1996b; 2001). The effect of all these changes leads to the accumulation of hydrogen peroxide, which further inhibits the catalase activity resulting in the destruction of melanocytes. Low epidermal catalase levels in both lesional and non-lesional epidermis of vitiligo patients suggests that the entire epidermis may be involved in this disorder (Schallreuter 1991). The cytotoxic effects of reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals on melanocytes was also studied (Morrone 1992; Thody 1991). Independent studies also showed that melanogenesis produces significant levels of ROS (Riley 1988). The present study suggests that environmental factors may account for the systemic origin of ROS in the susceptible patients.

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (Schallreuter et al 1991). In the present study significant increase in erythrocyte SOD activity was observed in all age groups of vitiligo patients compared to their respective controls (Figure 1). The 16-25 yrs age group showed the highest increase (66.93%, $p<0.0001$) in SOD activity compared to 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 (1996). However, Picardo et al (1994) reported that SOD activity

in erythrocytes of vitiligo patients was not significantly different from the healthy age matched controls (Picardo et al 1994).

Significant decrease in catalase (Figure 6) and glutathione peroxidase (Figure 3) activities was observed in vitiligo patients compared to the controls. Lower catalase activity has been observed by Maresca et al (1997) and Schallreuter et al (1991) (Maresca et al 1997; Schallreuter et al 1991). However, studies in vitiliginous melanocytes by other workers showed no change in catalase activity (Dell'Anna et al 2001; Beazley et al 1999). They also showed a significant decrease in GPX activity in vitiligo patients compared to controls (Dell'Anna et al 2001; Beazley et al 1999).

We observed significant decrease in glucose 6 phosphate dehydrogenase activity (Figure 4) in concordance with Saha et al (Saha et al 1982). G 6 PDH is the first rate- limiting enzyme in the hexose monophosphate shunt (HMS) pathway, playing an important role in the regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). 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 HMS pathway is the only source of NADPH, which is necessary to protect the cell from oxidative damage (Mehta 1991; Sodecinde 1992). Significantly lower GSH levels were observed in overall vitiligo patients ($p<0.001$) and 36-45 ($p<0.001$), 46-55 ($p<0.03$) yrs age groups of vitiligo patients compared to respective controls (Figure 5). Passi et al (1998) showed that epidermal GSH levels of active vitiligo patients were significantly lower compared to controls (Passi et al 1998). Changes in plasma vitamin E levels in the present study were non-significant (Figure 7) and our results are in concordance with Picardo et al (1994) (Picardo et al 1994). Vitamin E is the only lipid-soluble antioxidant concentrated mainly in plasma membrane and erythrocyte membranes. It terminates free

radical reactions acting as a chain breaking antioxidant. Significant increase in lipid peroxidation levels was also observed in all age groups of vitiligo patients, where 46-55 yrs age group showed 29.77 % ($p<0.0015$) increase compared to controls (Figure 2). However, non-significant changes in plasma lipoperoxides of vitiligo patients were reported by Picardo et al (1994).

Thus increased levels of erythrocyte SOD (Agrawal et al 2002) in vitiliginous patients could enhance the systemic production of H_2O_2. The downstream antioxidant enzymes that neutralize H_2O_2 are catalase and glutathione peroxidase. Significant decrease in catalase and GPX was observed in vitiligo patients compared to controls. Reduced catalase levels would not be able to scavenge the H_2O_2 produced by the SOD. As GPX also neutralizes lipid hydroperoxides, low levels of GPX in the 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 G 6 PDH), 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, G 6 PDH 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 indicating that melanocyte damage in vitiligo may be linked with generalized oxidative stress. Though increased oxidative stress is observed in the age group of 46 – 55 yrs, this is not statistically significant among the groups. No significant differences are observed in the antioxidant status between segmental and nonsegmental types of vitiligo as well as active and stable vitiligo. Thus,

systemic oxidative stress may have a pathophysiological role in all the age groups and types of vitiligo (Agrawal et al 2006).

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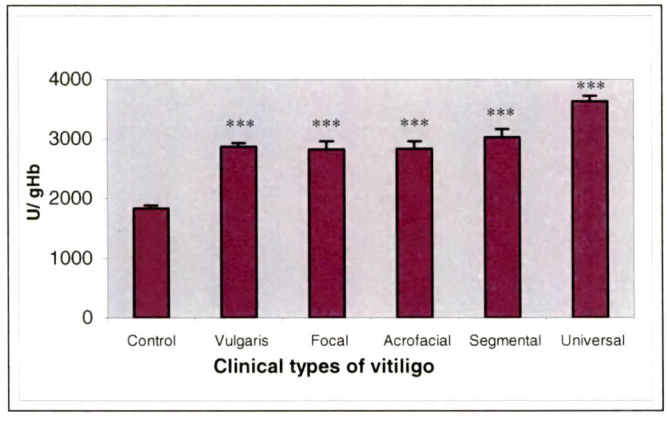
CHAPTER III B

THE ANTIOXIDANT STATUS OF VITILIGO PATIENTS OF DIFFERENT CLINICAL TYPES

3B.1. Results

The present study is an attempt to analyze the antioxidant status of different clinical types of vitiligo. Vitiligo patients were classified according to different clinical types i.e. vulgaris, focal, segmental, acrofacial and universal for the analysis of antioxidant status. Antioxidant enzyme activity, non-enzymatic antioxidants and oxidative damage of polyunsaturated fatty acids (LPO) in vitiligo patients and healthy controls were estimated. Segmental, vulgaris, acrofacial and focal types of vitiligo showed an increase in the erythrocyte SOD activity by 65.13%, 56.66%, 54.64% and 54.04% respectively compared to controls (Figure 1).

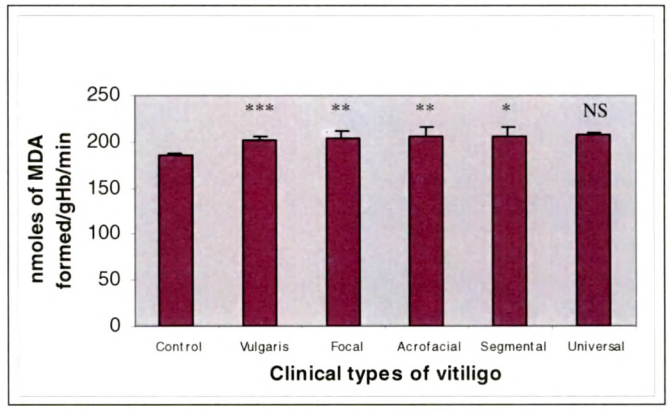
Figure 1. Superoxide dismutase activity in erythrocytes of controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. ***, $p<0.001$.

Similarly an increase in the LPO levels of segmental, vulgaris, acrofacial and focal types of vitiligo patients was observed i.e. 11.70%, 9.26%, 11.86% and 10.73% compared to controls (Figure 2).

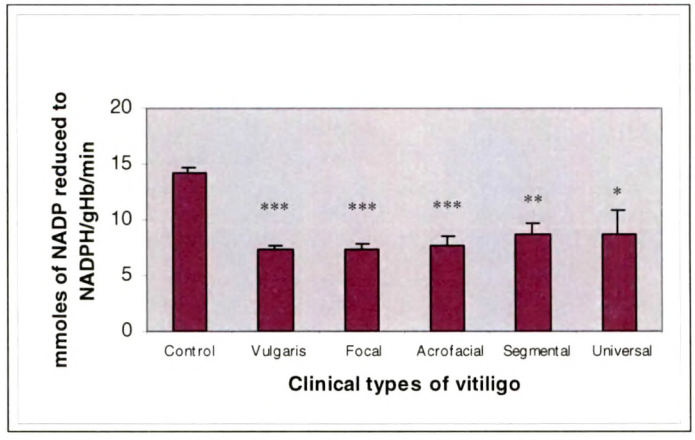
Figure 2. Erythrocyte lipid peroxidation levels in controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$, NS, non significant

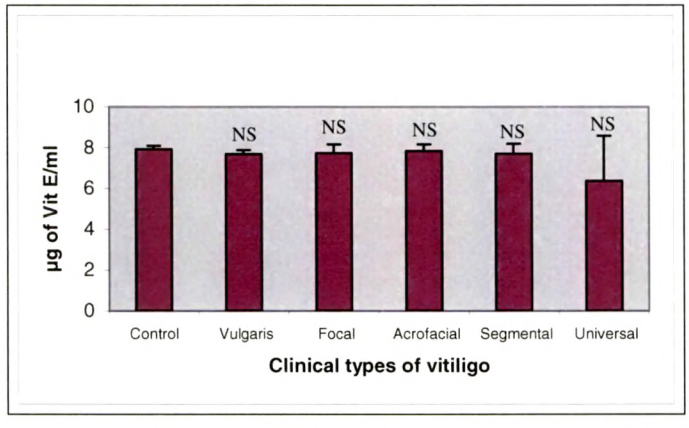
A significant decline in the G6PDH activity was observed in all types of vitiligo, i.e. of 48.44%, 48.65%, 45.26% and 39.17% in vulgaris, focal, acrofacial and segmental types respectively (Figure 3). However, vitamin E levels showed no significant change in different types of vitiligo compared to controls (Figure 4). A significant decrease in the catalase levels was found in focal and vulgaris types of vitiligo i.e. 17.30 % and 8.52% compared to controls (Figure 5). However, in segmental and acrofacial vitiligo, no significant change was observed in the catalase levels. A significant decrease in GPX was observed only in vulgaris (9.78%) and focal (13.94%) types of vitiligo compared to controls (Figure 6). No significant change in the GPX levels was observed in segmental and acrofacial vitiligo (Figure 6).

Figure 3. Glucose 6 phosphate dehydrogenase activity in erythrocytes of controls and different clinical types of vitiligo#



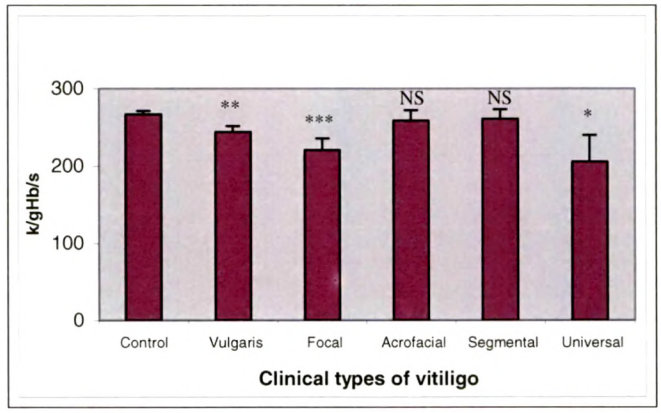
Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$, NS, non significant

Figure 4. Vitamin E levels in the plasma of controls and different clinical types of vitiligo#



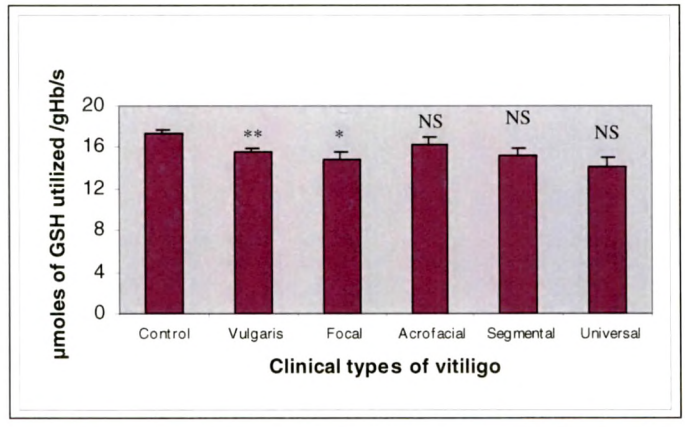
Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. NS, non significant

Figure 5. Catalase activity in erythrocytes of controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$, NS, non significant

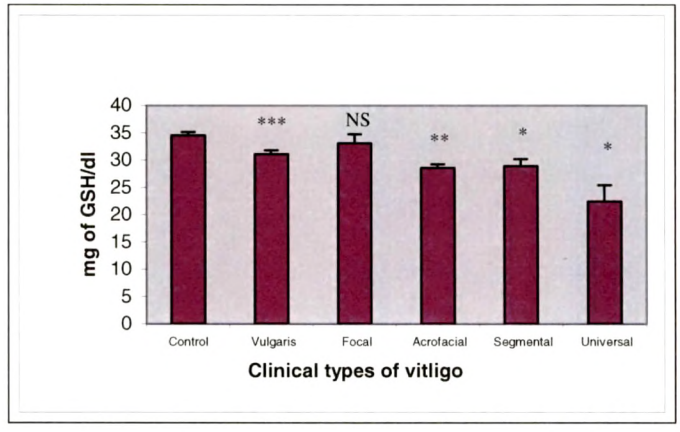
Figure 6. Glutathione peroxidase activity in erythrocytes of controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. *, $p<0.05$; **, $p<0.01$ and NS, non significant

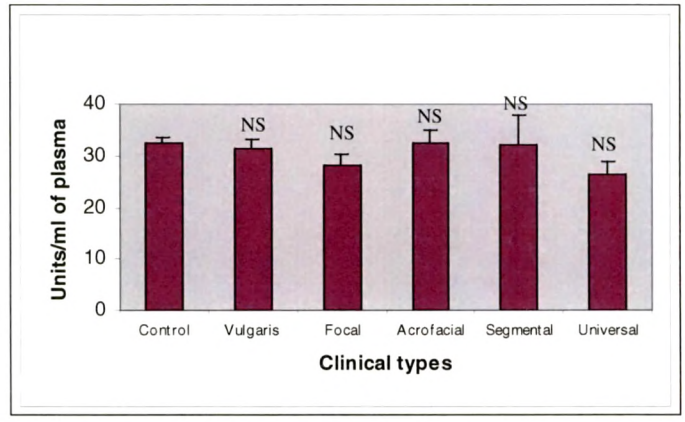
A significant decrease in whole blood GSH levels i.e. 10%, 17.17%, 16.18%, in vulgaris, acrofacial and segmental vitiligo was observed compared to controls (Figure 7). No significant change in the GSH levels was observed in focal vitiligo compared to controls (Figure 7). Also no significant change in GST and GR activities were observed in different clinical types of vitiligo (Figure 8 & Figure 9).

Figure 7. Whole blood GSH levels of controls and different clinical types of vitiligo#



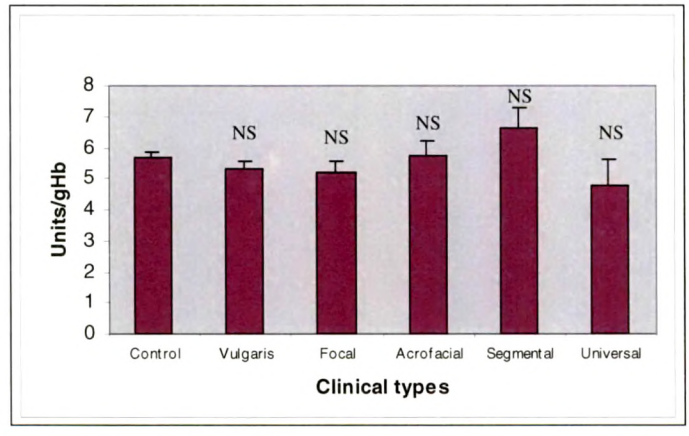
Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. *, $p<0.05$; **,$p<0.01$ and ***, $p<0.001$, NS, Non significant

Figure 8. Glutathione *S* transferase activity in the plasma of controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. NS, non significant

Figure 9. Glutathione reductase activity in the plasma of controls and different clinical types of vitiligo#



Values are given as mean ± SE of 126 individual observations in controls; 71 individual observations vulgaris vitiligo; 20 individual observations in focal vitiligo; 19 individual observations acrofacial vitiligo; 10 individual observations in segmental vitiligo and 4 observations in universal vitiligo respectively. NS, non significant

3B.2. Discussion

Oxidative stress acting as the triggering event in melanocyte degeneration is well established (Picardo et al 1994; Passi et al 1998). Different molecular events which lead to the accumulation of hydrogen peroxide are well documented (Schallreuter et al 1994; 1996; Rokos et al 2002; Gibson and Lilley 1997; Kaufman 1997; Dell'Anna et al 2001; Maresca et al 1997; Beazley et al 1999; Jimbow et al 2001; Schallreuter et al 1991). Hydrogen peroxide thus formed further inhibits epidermal catalase resulting in oxidative stress, which leads to the destruction of melanocytes (Schallreuter et al 1999). Low epidermal catalase levels in both lesional and non-lesional epidermis of vitiligo patients suggests that the entire epidermis may be involved in this disorder (Schallreuter et al 1999). Oxidative stress hypothesis also considers a systemic involvement during the course of the disease (Boisseu-Garsuad et al 2002; Picardo et al 1994; Yildirim et al 2003; Agrawal et al 2004). Thus oxidative stress could act as the initial triggering event in melanocyte degeneration. 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 (Taieb 2000).

Superoxide dismutase scavenges the superoxide radicals and reduces its toxicity (McCord and Fridovich 1969). In the present study significant increase in erythrocyte SOD activity was observed in all types of vitiligo patients compared to controls irrespective of the clinical types (Figure1). There are no specific reports available on the SOD levels in different clinical types of vitiligo. However, an increase in SOD activity was reported by Chakraborty et al (1996), Yildirim et al (2003) and Agrawal et al (2004). Picardo et al (1994) reported that SOD activity in erythrocytes of vitiligo patients was not significantly different from the healthy age matched controls (Picardo et al 1994). Significant decrease in serum SOD levels was reported by Koca et al (2004) in generalized vitiligo (Koca et al 2004).

Passi et al (1998) showed that there is no significant change in the epidermal SOD levels in vitiligo patients compared to controls (Passi et al 1998). Maresca et al (1997) have observed no difference in the SOD activity in cultured vitiliginous melanocytes compared to cultured melanocytes of normal subjects (Maresca et al 1997).

Catalase activity was found to be significantly lower in vulgaris and focal types while no significant change in the catalase activity was observed in other clinical types i.e. acrofacial and segmental vitiligo (Figure 5). There is no report on the catalase levels in different clinical types of vitiligo. However, studies in vitiliginous melanocytes showed lower catalase activity compared to normal melanocytes from controls (Maresca et al 1997; Schallreuter et al 1991). No significant change in the erythrocyte catalase activity was also reported (Dell'Anna et al 2001; Beazley et al 1999).

GPX converts H_2O_2 and other peroxides into water (Halliwel et al 1992). In the present study significant decrease in GPX levels was observed in vulgaris and focal types only and no change was observed in segmental and acrofacial types of vitiligo (Figure 6). Beazley et al (1999) and Yildirim et al (2003) reported low levels of GPX in the blood of generalized vitiligo patients compared to controls and there are no reports on other clinical types of vitiligo. However Picardo et al (1994) and Passi et al (1998) reported no differences in erythrocyte GPX levels in vitiligo patients compared to controls (Picardo et al 1994; Passi et al 1998).

We observed significant decrease in glucose 6-phosphate dehydrogenase activity in all types of vitiligo irrespective of the clinical types (Figure 3). 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 to controls. G6PDH is the first rate- limiting enzyme in the hexose monophosphate shunt (HMP) pathway, playing an important role in the regeneration of the reduced form of nicotinamide adenine dinucleotide

phosphate (NADPH). 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 the red cells HMP pathway is the only source of NADPH, which is necessary to protect the sulfhydryl (-SH groups) of proteins from the oxidative damage (Mehta 1991; Sodecinde 1992). There are reports showing that G6PDH inhibitors potentiated H_2O_2 induced cell death and over expression of G6PDH increased resistance to H_2O_2 induced cell death (Tian et al 1999).

Significantly lower GSH levels were observed in vulgaris, acrofacial and segmental types of vitiligo (Figure 7). 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 to 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 to 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 all types of vitiligo (Figure 4). 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. Vitamin E is the only lipid-soluble antioxidant concentrated mainly in plasma membrane and erythrocyte membranes. It terminates free radical reactions acting as a chain breaking antioxidant.

Significant increase in lipid peroxidation levels was observed in all clinical types of vitiligo except universal type (Figure 2, Table 1). The non-significant change in LPO levels in universal type of vitiligo may be because of less number of patients in this study, (n=4). There is no report on the LPO levels in different

clinical types of vitiligo. However, 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 all clinical types of vitiligo compared to 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 and glutathione peroxidase were found to be decreased significantly in vitiligo patients. Low levels of catalase or/and GPX in patients could result in excessive production of H_2O_2, which in turn leads to oxidative stress. Plasma vitamin E levels of these 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. Thus low levels of catalase, GPX, G6PDH and GSH could contribute to oxidative stress as is evident by high LPO levels in vitiligo patients.

Elevated LPO levels in all clinical types of vitiligo (Figure 2) 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. In focal type of vitiligo, GSH and plasma vitamin E levels are unchanged and other antioxidant parameters are same as in vitiligo vulgaris. Acrofacial and segmental types of vitiligo showed the same pattern of change in the enzymatic and non-enzymatic antioxidants. In universal type of vitiligo there is no significant increase in the LPO levels even though significant decrease in the catalase, G6PDH, GSH and significant increase in the SOD levels was observed. The summary of overall results is given in the Table 1.

Table 1. Summary of the antioxidant status in different clinical types of vitiligo compared to controls

	Segmental	Vulgaris	Acrofacial	Focal	Universal
LPO	↑	↑	↑	↑	NS
SOD	↑	↑	↑	↑	↑
CAT	NS	↓	NS	↓	↓
GPX	NS	↓	NS	↓	NS
G6PDH	↓	↓	↓	↓	↓
GST	NS	NS	NS	NS	NS
GR	NS	NS	NS	NS	NS
GSH	↓	↓	↓	NS	↓
Vit E	NS	NS	NS	NS	NS

↑, increased; ↓, decreased; NS, non significant

In conclusion, impairment of the systemic antioxidant system resulting in oxidative stress in all types of vitiligo has been observed in this study indicating that melanocyte damage in vitiligo may be linked to generalized oxidative stress. The present study is the first report on the antioxidant parameters of different clinical types of vitiligo in Baroda, Gujarat state, India (Shajil and Begum 2006).

3B.2. References

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