CHAPTER V

EVALUATION OF OXIDATIVE STRESS AND AUTOIMMUNE HYPOTHESES IN ONSET AND PROGRESSION OF VITILIGO

5.1 INTRODUCTION

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from the epidermis. The mechanism of melanocyte loss from the epidermis to cause vitiligo is not yet clearly understood (Taieb *et al.*, 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are oxidative stress, autoimmune and neurochemical hypotheses (Ortonne and Bose, 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp *et al.*, 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp *et al.*, 2001). Also the immune suppressive effect of a number of repigmenting therapies (steroids) indirectly supports the autoimmune mediated process of depigmentation (Ongenae *et al.*, 2003). However, the exact mechanism involved in vitiligo pathogenesis remains obscure.

The aim of this study was to find whether oxidative stress or autoimmunity plays a major role for the initiation of vitiligo pathogenesis and its progression. Lipid peroxidation levels, the index of oxidative stress was assessed to evaluate the oxidative stress hypothesis and the levels of antimelanocyte antibodies were measured to evaluate the autoimmune hypothesis in patients with the onset of vitiligo (<3 months) and compared with the patients suffering from the disease for long duration of (>3 months). For the progression of the disease, patients with active vitiligo and stable vitiligo were compared for LPO levels and antimelanocyte antibody levels.

5.2 MATERIALS AND METHODS

5.2.1 Study subjects:

The study group included 300 vitiligo patients and comprised of 138 males and 162 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 400 ethnically sex-matched unaffected individuals (188 males and

212 females) were included in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo or any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 300)	(n = 400)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	138 (46.0%)	188 (47.00%)
Female	162 (54.0%)	212 (53.00%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Patients with <3 months onset	124 (41.33%)	NA
>3 months onset	176 (58.67%)	NA
Active vitiligo	215 (71.67%)	NA
Stable vitiligo	85 (28.33%)	NA
Family history	41 (13.66%)	NA

5.2.2 Estimation of lipid peroxidation (LPO) levels:

Erythrocyte LPO levels were estimated according to Beuge and Aust (1978) method as described in Chapter 2.

5.2.3 Estimation of antimelanocyte antibody levels by enzyme linked immunosorbent assay (ELISA):

In the present study, plasma from vitiligo patients was examined for the reactivity with the human melanoma cell line (SK Mel 28) to find the levels of antimelanocyte antibodies in vitiligo patients compared to controls. We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA) and plasma was extracted. Human melanoma cell line SK Mel 28 was obtained from NCCS, Pune and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mg/L gentamycin at 37°C under 5% CO2 in a humidified atmosphere (Hann and Kim, 1995).

Cells were harvested by scraping and lysed by adding lysis buffer (HEPES 20 mM, EGTA 1 mM, PMSF 1 mM, MgCl2 1.5 mM, NaCl 150 mM, CuSO4 1mM, Trition -X 100 1%, Glycerol 1%). Protein estimation in the lysate was done by Lowry's method (Lowry *et al.*, 1951). Fifty µl of the cell lysate containing 1 µg protein was loaded in each well of the microtiter plate and kept at 4°C overnight. Excess antigen was discarded, washed 3 times with PBS, blocked with 1% BSA in PBS and incubated for 1 hour. Excess blocking reagent was discarded and washed 3 times with PBS. Fifty µl of plasma (1:10,000 diluted in PBS) was added to the micro titer plates and incubated for 2 hours at room temperature. Excess plasma was discarded and washed 3 times with PBS containing 0.2% Tween 20. Added 50 µl of 1: 2000 diluted secondary antibody (Rabbit anti human IgG HRP conjugate, Bangalore Genei, India) and incubated for 1 hour. Excess antibody was discarded and washed 3 times with PBS containing 0.2% Tween 20. Fifty µl substrate (TMB-H_2O_2) was added and incubated for 5 minutes for the color development. The reaction was stopped by adding 200 µl of 1N H_2SO_4 as the color developed and OD was read at 405 nm.

5.2.4 Statistical analysis:

LPO and antimelanocyte antibody levels were plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.05 were considered as statistically significant.

5.3 RESULTS

Antimelanocyte antibody and LPO levels were measured in 300 vitiligo patients and 400 controls. Vitiligo patients showed significant increase in LPO levels and antimelanocyte antibody levels as compared to controls (p=0.009; p<0.0001 respectively) (Figure 1A & B).

Further, we compared lipid peroxidation levels to evaluate the oxidative stress hypothesis and antimelanocyte antibody levels to evaluate the autoimmune hypothesis in patients at the onset of vitiligo (< 3 months) and with those suffering from vitiligo for a long duration (>3 months). Significant low (p=0.0008) levels of antimelanocyte antibodies were observed in <3 months patients compared to >3 months patients (Figure 2B). On the contrary, significant high (p=0.009) LPO levels were observed in vitiligo patients at the onset (>3 months) compared to vitiligo patients with long duration of the disease (Figure 2A).

Moreover, analysis of antimelanocyte antibody levels and LPO levels were monitored based on progression of the disease i.e. active vitiligo and stable vitiligo. Interestingly, patients with active vitiligo showed significant increase in antimelanocyte antibody (p<0.0001) and LPO levels (p=0.002) as compared to stable vitiligo patients (Figure 2C & D).

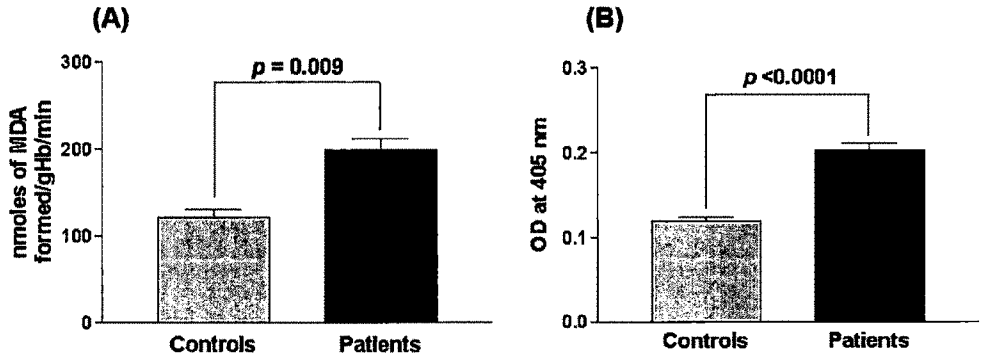

Figure 1. Lipid peroxidation (LPO) and antimelanocyte antibody levels in controls and vitiligo patients:

(A) LPO levels in 300 vitiligo patients and 400 controls. Vitiligo patients showed significantly increased LPO levels as compared to controls (Mean ± SEM: 199.5 ± 12.84 vs 120.7 ± 9.199; p<0.0001).

(B) Antimelanocyte antibody levels in 300 vitiligo patients and 400 controls. Vitiligo patients showed significantly increased antimelanocyte antibody levels as compared to controls (Mean ± SEM: 0.2029 ± 0.0081 vs 0.1186 ± 0.0042; $p<0.0001$).

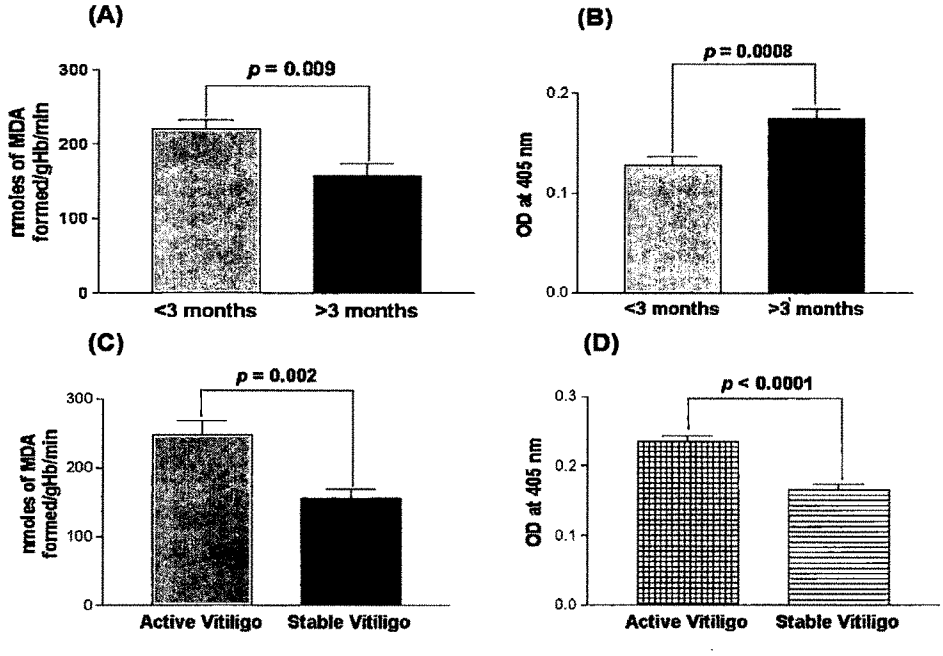

Figure 2. Lipid peroxidation (LPO) and antimelanocyte antibody levels with respect to onset and progression of disease:

(A) LPO levels in vitiligo patients with <3 months onset (n=124) and >3 months (n=176) of disease. Patients with <3 months onset showed significantly higher levels of LPO as compared to >3 months onset (Mean ± SEM: 221.1 ± 11.65 vs 158.5 ± 15.51; p=0.009).

(B) Antimelanocyte antibody levels in vitiligo patients with <3 months onset (n=124) and >3 months (n=176) of disease. Patients with <3 months onset showed significantly lower levels of antimelanocyte antibody as compared to >3 months of disease (Mean ± SEM: 0.1280 ± 0.0084 vs 0.1743 ± 0.0097; p=0.0008).

(C) LPO levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of LPO as compared to stable vitiligo (Mean ± SEM: 247.7 ± 20.94 vs 155.1 ± 13.84; p=0.002).

(D) Antimelanocyte antibody levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of antimelanocyte

antibody as compared to stable vitiligo (Mean ± SEM: 0.2350 ± 0.0086 vs 0.1653 ± 0.0079; $p<0.0001$).

5.4 DISCUSSION

There are several reports on the antimelanocyte antibody levels in vitiligo patients compared to controls. However, no study has been performed to evaluate both oxidative stress and autoimmune hypotheses simultaneously at the onset of vitiligo to find out which factor/s play a major role in triggering vitiligo. In addition, it is also not clear which hypothesis is prevailing in understanding the progression of the disease.

Naughton *et al.,* (1983) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation (Naughton *et al.,* 1983a). In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton *et al.,* 1983b). However, Grimes *et al.,* (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Farrokhi *et al.,* (2005) also showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients.

The present study reports that patients with long duration (>3 months) exhibited significantly lower (p=0.009) LPO levels compared to patients with early onset (<3 months) of the disease (Figure 2A). However, patients at the onset (<3 months) showed significantly lower (p=0.0008) levels of antimelanocyte antibodies compared to patients with long duration (>3 months) of vitiligo (Figure 2B). These results suggest that oxidative stress rather than autoantibodies plays a major role in the initiation of vitiligo pathogenesis. Moreover, our results on neurochemical hypothesis also showed that significant decrease in acetylcholine esterase levels in vitiligo patients compared to controls (Shajil *et al.,* 2006). This could be due to H_2O_2 mediated oxidation of AChE, thus emphasizing the role of oxidative stress in precipitating vitiligo (Schallreuter *et al.,* 2004). Interestingly, analysis of antimelanocyte antibody levels and LPO levels were monitored based on progression of the disease revealed that both LPO and antimelanocyte antibodies were higher in active cases of vitiligo compared to stable vitiligo (Figure 2C & D). This signifies the equal contribution of both oxidative stress and autoimmunity in disease progression.

There are several ways by which high oxidative stress, besides having a direct melanocytotoxicity, can induce an autoimmune attack against melanocytes. The structures of melanocytic macromolecules such as Melan-A and tyrosinase, may markedly change by acute or chronic oxidative stress and can act as antigens (neo-antigens). Neo-antigens with sufficient homology or identity to host antigenic proteins induce auto-reactivity. This phenomenon is referred to as 'molecular mimicry' (Kannan, 2006). Aldehydic products, mainly the 4-hydroxy-2-alkenals, form adducts with proteins and make them highly immunogenic (Kurien *et al.*, 2006). Hydroxyl radicals are also very highly reactive and could attack a wide range of targets. The presence of lesions in vitiligo patients can be explained by this mechanism (Vahedi Darmian *et al.*, 2004). Over time, chronic oxidative stress could generate several adducted molecules that could act as 'neo-antigens'. This is consistent with the slow maturation of auto-antibodies during the evolution of the disease. More neo-antigens are involved in amplifying the autoaggressive lymphocytes by a process referred to as 'antigen spreading'. This is an autoimmune reaction initially directed against a single autoantigen that spreads to other autoantigens, causing the T helper cells to recognize them (Kannan, 2006).

During chronic oxidative stress, neo-antigens potentially cause tissue damage and release a plethora of sequestered auto-antigens. This process is referred to as the 'bystander effect'. Such an outburst of auto-antigens from the target tissue would potentially amplify the effect of the neo-antigens, leading to the breakdown of self-tolerance (Kannan, 2006).

In conclusion, this study shows that oxidative stress is the initial triggering event to precipitate vitiligo in Gujarat population which is then exacerbated by contribution of autoimmune factors together with oxidative stress.

5.5 REFERENCES:

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