6.1 INTRODUCTION

Oxidative stress has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocytes destruction (Laddha *et al*., 2013) and is marked by the accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al*., 2001). Our previous studies have suggested a significant increase in oxidative stress in vitiligo patients which is evident by high lipid peroxidation levels and an imbalance in the antioxidant enzymes (Laddha *et al*., 2013; Shajil, and Begum, 2006; Mansuri *et al*., 2016; Mansuri *et al*., 2017). Recent studies show that Poly (ADP-ribose) polymerase 1 (PARP-1), one of the major players of ROS-induced cell death, is involved in regulation of tissue inflammation, autoimmunity and apoptosis etc. (Kyeong *et al.*, 2012). PARP-1 is a DNA repair enzyme that catalyses the poly (ADP-ribosyl)ation of proteins using NAD^+ as its substrate. PARP1 is activated 100- fold by DNA strand breaks and has a major role in base excision repair pathway (Caldecott, 2008). PARP-1 is known to have a role in the regulation of expression of various proteins that are implicated in the inflammation at the transcriptional level [(e.g., inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), [COX-2, and major histocompatibility complex class II (MHC Class II)]. The absence of functional PARP-1 (either genetic or pharmacological) decreased the expression of a host of pro-inflammatory mediators, including cytokines, chemokines, and adhesion molecules. The absence of PARP-1 also reduced tissue infiltration with activated phagocytes in experimental models of inflammation, circulatory shock, and ischemia reperfusion (Szabo, 2006). Studies have shown that PARP-1 is overexpressed in a variety of cancers and has been linked to the prognosis of cancers, most notably breast cancer. These seminal observations have been extended to show that PARP-1 further participates in the activation of other essential pro-inflammatory signaling cascades (Ha *et al*., 2002). A variety of endogenous and exogenous stress signals including those generated by oxidative, genotoxic, thermal, oncogenic, metabolic, and inflammatory stresses can trigger responses from PARP-1 leading to pathological conditions such as cancer, inflammation related diseases, autoimmune diseases, neurodegenerative diseases, and metabolic disorders.

Oxidative stress triggers extensive DNA damage, over activation of PARP and consequent depletion of the cellular NAD^+ levels impairing glycolysis, Krebs cycle, mitochondrial electron transport and eventually resulting in ATP depletion and consequent cell dysfunction and death by necrosis. During conditions of PARP over activation, pharmacological inhibition of PARP or genetic deletion of PARP-1 preserves cellular NAD^+ and ATP pools in oxidatively stressed cells, thereby allowing them to function normally, otherwise, if the apoptotic process has initiated it utilizes the apoptotic machinery and die by apoptosis instead of necrosis (Fiorillo *et al.*, 2006; Levrand *et al*. 2006). The inhibition of PARP by its inhibitors may offer therapeutic benefit in various disease conditions by preventing acute cell death. The ability of PARP inhibitors to suppress the expression of pro-inflammatory genes may be further exploited in various treatment modalities associated with acute and/or chronic inflammation (e.g., atherosclerosis, cardiovascular aging), inflammatory diseases, and various forms of cancer. 1,5-dihydroxyisoquinoline (DHQ) is a well characterized PARP inhibitor and was found to inhibit the PARP activity completely at non-cytotoxic concentrations (Shah *et al*., 1996). The recent clinical availability of the PARP inhibitors opens the door for their potential therapeutic use for various diseases. However, the role of PARP-1 activation and role of NFκB under oxidative stress in melanocytes has not been elucidated yet.

Therefore, in the present study, we aimed to monitor the effect of H_2O_2 on NHM and their rescue from H_2O_2 induced cell death using DHQ by assessing PARP1 activation and PARylation status. Moreover, we also monitored the effect of oxidative stress on NHM for MITM-M, *TYR*, and *ICAM1* expression.

6.2 MATERIALS AND METHODS

6.2.1 Ethics statement & Culture establishment of primary normal human melanocytes (NHM)

These aspects are described in Chapter II.

6.2.2 Trypan blue exclusion assay

For cell viability assay, NHM were pretreated with 100 μM DHQ for 4 hrs, followed by 15 min exposure of H_2O_2 (0, 100, 250, 500 μM) along with media change**.** In this test, the cell suspension is mixed with trypan blue dye (1:10 dilution) and then visually examined under light microscope to distinguish whether cells take up or exclude dye. (Strober *et al.*, 2001).

6.2.3 Western Blot analysis

To detect alterations in the protein levels, NHM were pre-treated with 100μM DHQ for 4 hrs, followed by a 15 min exposure of H_2O_2 (100 μM) and then cells were scraped on ice for cell harvesting. Total cell lysates were obtained by sonication (Using microprobe, 20% amplitude) using Laemmli Buffer (2% SDS, 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, and 5% 2-β-mercaptoethanol) extraction. Thirty µg of total protein were subjected to electrophoresis in 10% and 6% SDS PAGE under reducing conditions for PARP-1 cleavage and PARylation respectively. Proteins were transferred to PVDF membrane and were blocked by blocking buffer (5% W/V BSA in PBS-T) for one hr. This was followed by probing Anti-PAR mouse mAb (10H) (Calbiochem, Germany) and Anti-PARP1rabbit antibody (cell signaling technology) overnight at 4ºC. Membrane was then probed with Peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) and Peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma) respectively, followed by six PBS-T and two PBS washes. Protein bands were detected by enhanced chemiluminescence system ECL (Amersham Pharmacia Biotech).

6.2.4 RNA isolation, cDNA synthesis and gene expression analysis

The transcript levels of different genes were estimated by semiQ PCR, using gene specific primers (Eurofins, Bangalore, India) as shown in Table 1. Expression of *GAPDH* gene was used as a reference. Details are described in Chapter IV.

Table 1. Primer sequences used for gene expression analysis.

Primer	**Sequence (5' to 3')**	**Amplicon size (bp)**	**Annealing Temperature (^{0}C)**
ICAM1FP	TCTGTTCCCAGGACCTGGCAATG	282	65

ICAM1RP	GGAGTCCAGTACACGGTGAGGAAG		
MITFM FP	CAAATGATCCAGACATGCGCTGG	180	61
MITFM RP	CTCGAGCCTGCATTTCAAGTTCC		
TYRFP	AGCACCCCACAAATCCTAACTTAC	92	63
TYRRP	ATGGCTGTTGTACTCCTCCAATC		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65
GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

'FP': forward primer; 'RP': reverse primer; 'bp': base pair.

6.2.5 Monitoring MITF-M levels upon H_2O_2 treatment by immunofluorescence

Details are described in Chapter IV.

6.2.6 Statistical analyses

All experiments were performed in triplicates using different batches of cells. Data are presented as the mean ± standard deviation (SD) and the statistical difference between two groups was analyzed by Student's t-test using GraphPad Prism version 3.02 software (GraphPad Software Inc. San Diego, CA, USA). *p*-values less than 0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Effect of PARP inhibitor (DHQ) on H_2O_2 induced NHM death:

Primary melanocytes were treated with different concentrations of H_2O_2 (0, 100, 250, 500 µM) for 15 min and incubated at 37ºC in humidified CO_2. NHM exhibited significant dose dependent decrease in the NHM viability upon H_2O_2 treatment (100, 250, 500 µM H_2O_2; p=0.0248, p= 0.0014 and p<0.0001 respectively Figure 1A). Further, we studied the dose-dependent effect of DHQ on NHM. NHM were treated with 0, 50, 100, 200 µM DHQ for 24 hours, followed by a cell count using trypan blue exclusion assay.

Concentration of DHQ up to 200 μM for 24 hours did not show any significant effect on cell morphology (Figure 1) and viability of NHM (Figure 2B). Therefore 100μM of DHQ was chosen for further experiments. In addition, we studied the effect of rescue of NHM death by PARP1 inhibitor in the presence and absence of H_2O_2. NHM were pre-treated with 100μM DHQ for 4 hours, followed by a 15 min exposure of H_2O_2 (0, 100, 250, 500 μM). Cell count was taken, using trypan blue exclusion assay. Pre-treatment with 100μM DHQ showed significant rescue from the cytotoxic effects of H_2O_2 both on cell morphology (Figure 1) and cell viability (Figure 2C). Significant rescue was observed at 100 μM, 250μM and 500μM of H_2O_2 (p=0.0471, p=0.0022 and p=0.0002 respectively).

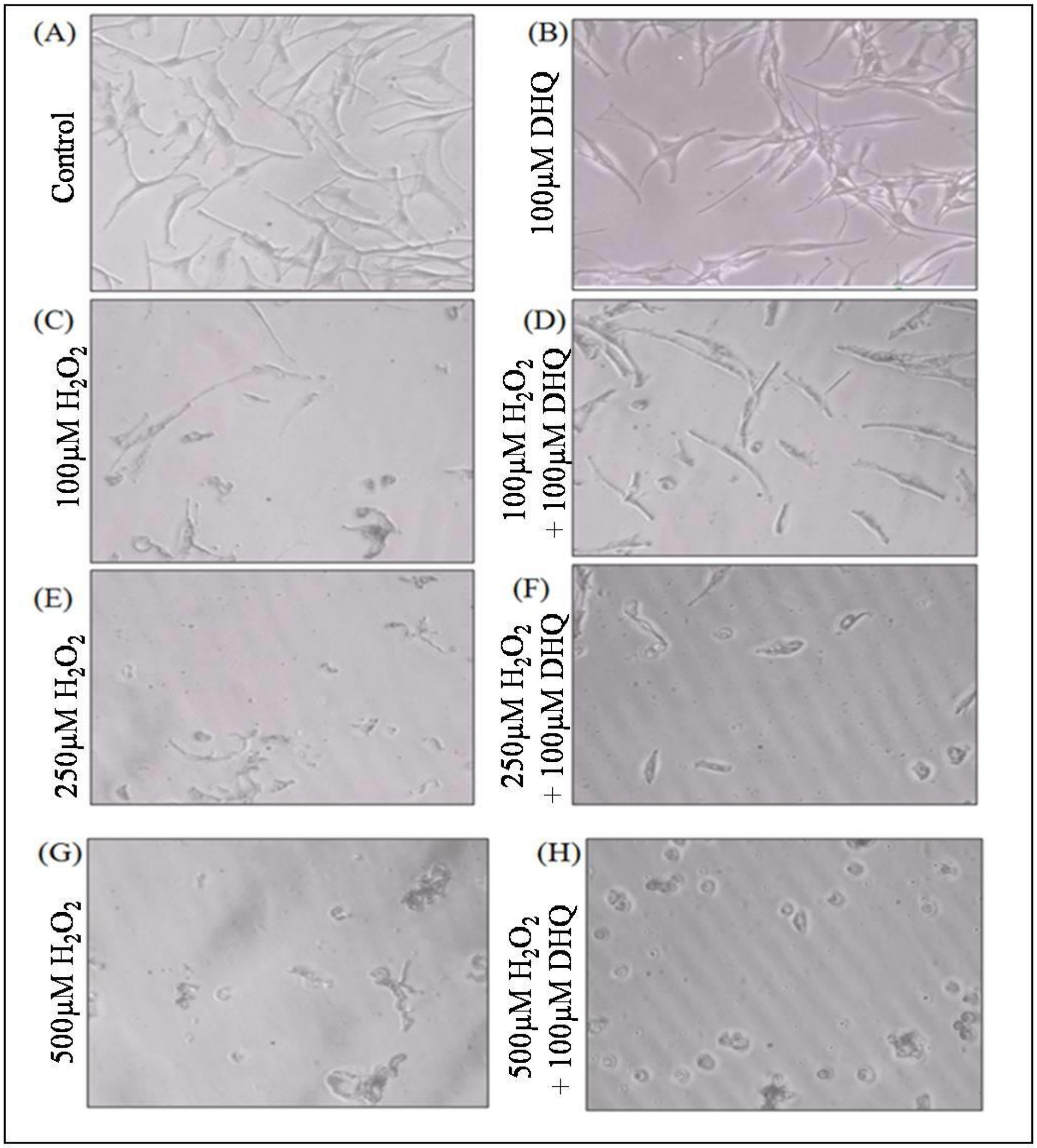


Figure 1: Morphological effect of DHQ on rescue of H_2O_2 induced NHM death: NHM were treated with respective doses as mentioned in figure (A) Control (B) 100μM DHQ (C) 100μM H_2O_2 (D) 100μM H_2O_2+ 100μM DHQ (E) 250μM H_2O_2 (F) 250μM H_2O_2+ 100μM DHQ (G) 500μM H_2O_2 (H) 500μM H_2O_2 + 100μM DHQ. Significant rescue was observed from dose dependent H_2O_2 mediated cell death upon pretreatment of DHQ.

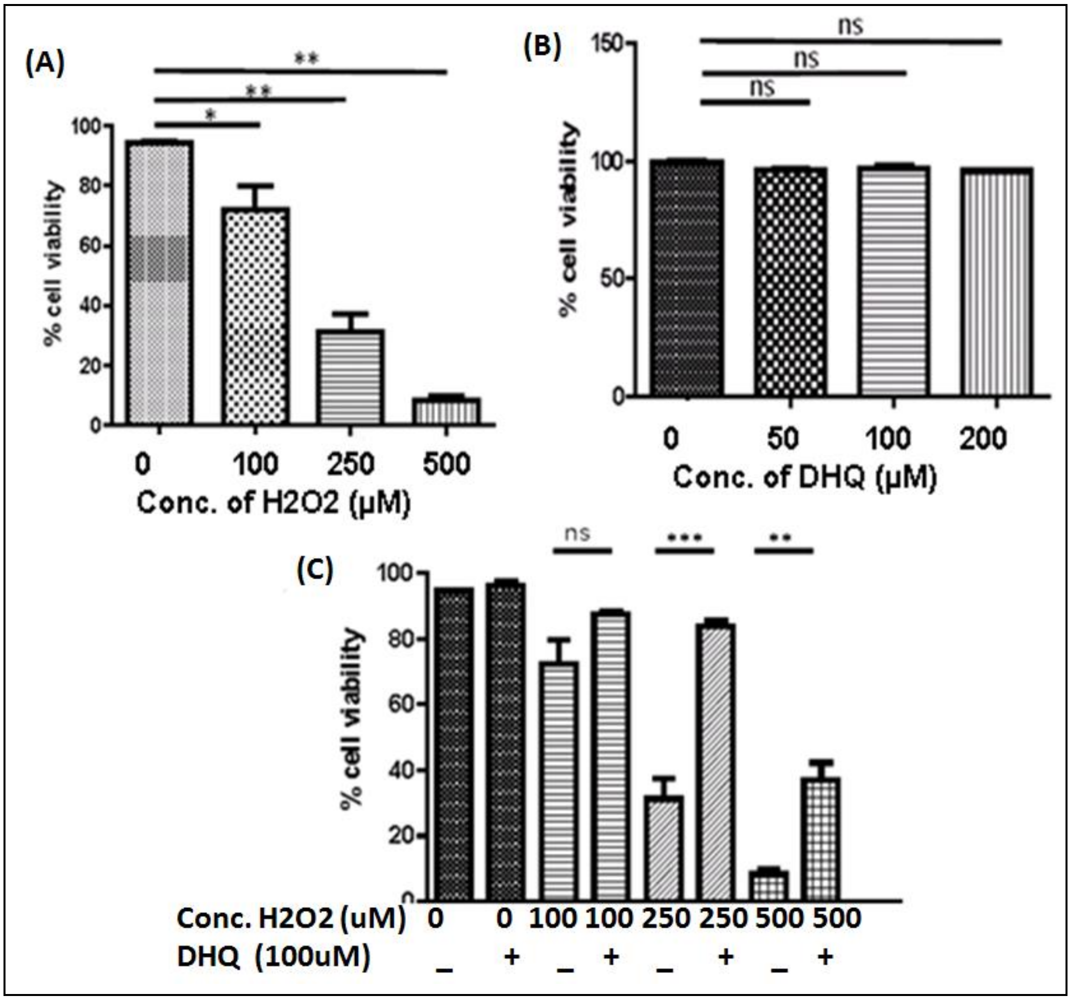


Figure 2: Effect of DHQ on rescue of H_2O_2 induced NHM death: (A) NHMs were treated with different doses of H_2O_2 (100µM, 250µM and 500µM) for 15 minutes followed by media change and cell viability was assayed by tyrpan blue exclusion assay. The results showed significant cell death (p=0.0248, p=0.0027 and p=0.0021 respectively; n=3).

(B) The cells were treated with different concentration of DHQ (50µM, 100 µM and 200µM) for 24 hours for cell viability analysis was performed by trypan blue assay. Dose dependent effect of DHQ on NHM viability by tyrpan blue exclusion showed no significant effect (n=3).

(C) Dose-dependent effect of H_2O_2 on NHM viability with and without pre-treatment (4 hours) of 100 µM DHQ. NHM were given 4 hours pretreatment with DHQ followed by

H_2O_2 exposure for 15 minutes. Media change was given after H_2O_2 treatment and cell count was taken by trypan blue exclusion assay. Significant rescue in cell death was observed at higher doses of H_2O_2 (p=0.0471; p=0.0002; p=0.0022); the values represent mean ±S.D. of three independent experiments.

6.3.2 Western blot analysis of PARylation and PARP1 activation upon DHQ mediated rescue from H_2O_2 induced NHM death:

NHM were treated with 100 µM H_2O_2 with and without DHQ for 15 min at 37ºC in humidified CO_2. DHQ exhibited significant restoration of a few NHM proteins which were degraded by H_2O_2 treatment. To confirm the same, we probed the samples (Control, 100µM H_2O_2, 100µM DHQ, 100µM DHQ +100µM H_2O_2) with anti-10H (PAR) antibody and PARP-1 antibodies. Our results demonstrated significant suppression of PARylation as well as PARP-1 activation upon DHQ treatment (Figures 3A and 3B). Also, NHM exhibited significant restoration of PARylation pattern of various proteins along with PARP-1 activation in DHQ + H_2O_2 group as compared to H_2O_2 group (Figures 3A and 3B). At higher concentration i.e., above 100µM of H_2O_2 there was complete degradation of proteins (data not shown).

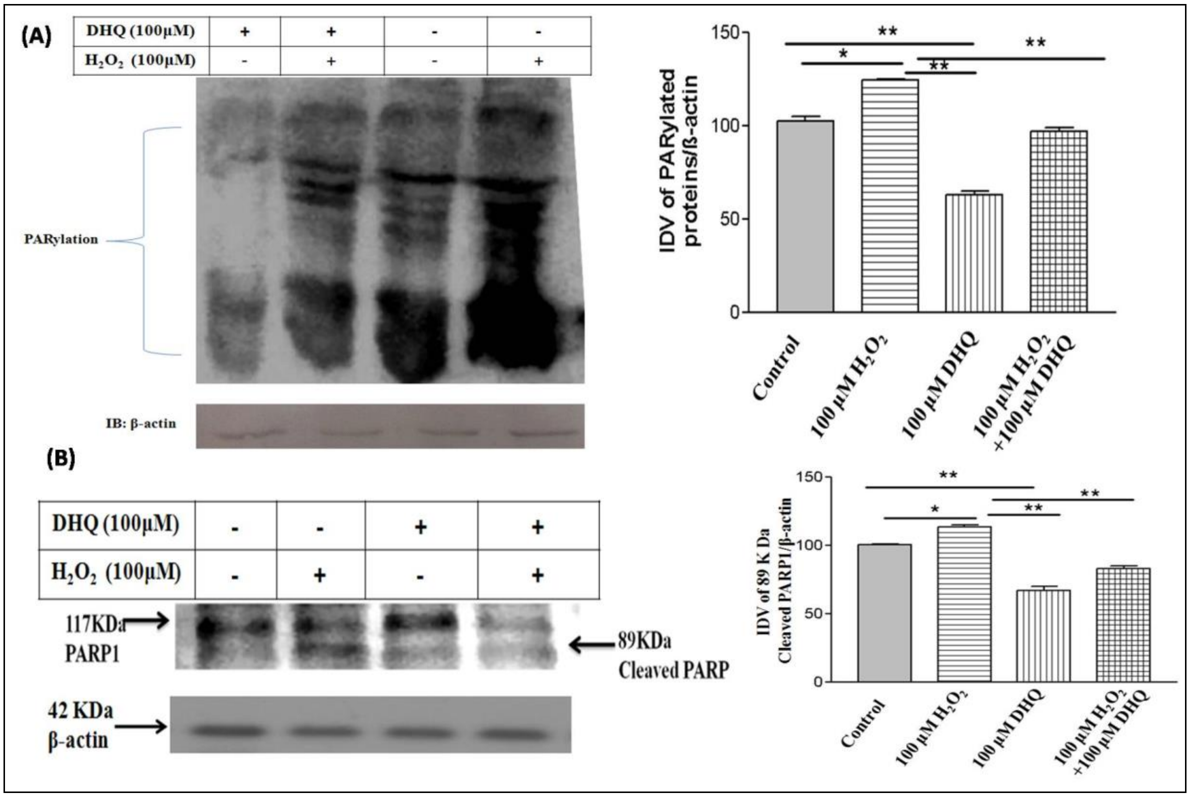


Figure 3: Analysis of PARylation and PARP1 activation upon DHQ mediated rescue from H_2O_2 induced NHM death: (A) Restoration of PARylation pattern of various proteins in DHQ+H_2O_2 group as compared to H_2O_2 group. DHQ treatment exhibited suppression of PARylation as compared to control group. Densitometric analysis for the DHQ and DHQ+H_2O_2 group reveled significant difference (p=0.0076) in terms of PARylation suppression.

(B) PARP-1 hyper activation was observed in H_2O_2 treated group (89 KDa cleaved fragment) which showed restoration in PARP1 inhibited conditions (DHQ + H_2O_2 group) as compared to H_2O_2 group. DHQ treatment suppressed PARP-1 activation. Densitometric analysis for the DHQ and DHQ+H_2O_2 group reveled significant difference (p=0.0062) in terms of PARP1 activation. β-actin was kept as a protein loading control for all western analysis (n=3).

6.3.3 Effect of oxidative stress on *MITF-M, TYR* and *ICAM-1* expression:

NHM were treated with 50 µM and 100 µM of H_2O_2 for 24 hrs. Significant decrease in *MITF-M* transcript levels was observed at both concentrations of H_2O_2 (p=0.0083 and p=0.0383 respectively) (Figure 4A). TYR is one of the target genes for MITF-M therefore we monitored the expression levels of *TYR*. Significant decrease in *TYR* transcript levels was observed with 50 µM and 100 µM of H_2O_2 (p=0.0439 and p=0.0109 respectively) (Figure 4B). However, there was no significant difference for *ICAM-1* transcript levels when treated with 50 µM and 100 µM of H_2O_2 (p=0.0772 and p=0.1325 respectively) (Figure 4C). We have also monitored effect of H_2O_2 on MITF-M protein expression in NHM**.** NHM upon treatment with 50 µM and 100 µM of H_2O_2 for 24 hrs significant decrease in MITF-M expression was observed in a dose dependent manner (p=0.0024 and p=0.0001 respectively) (Figures 5 A and B).

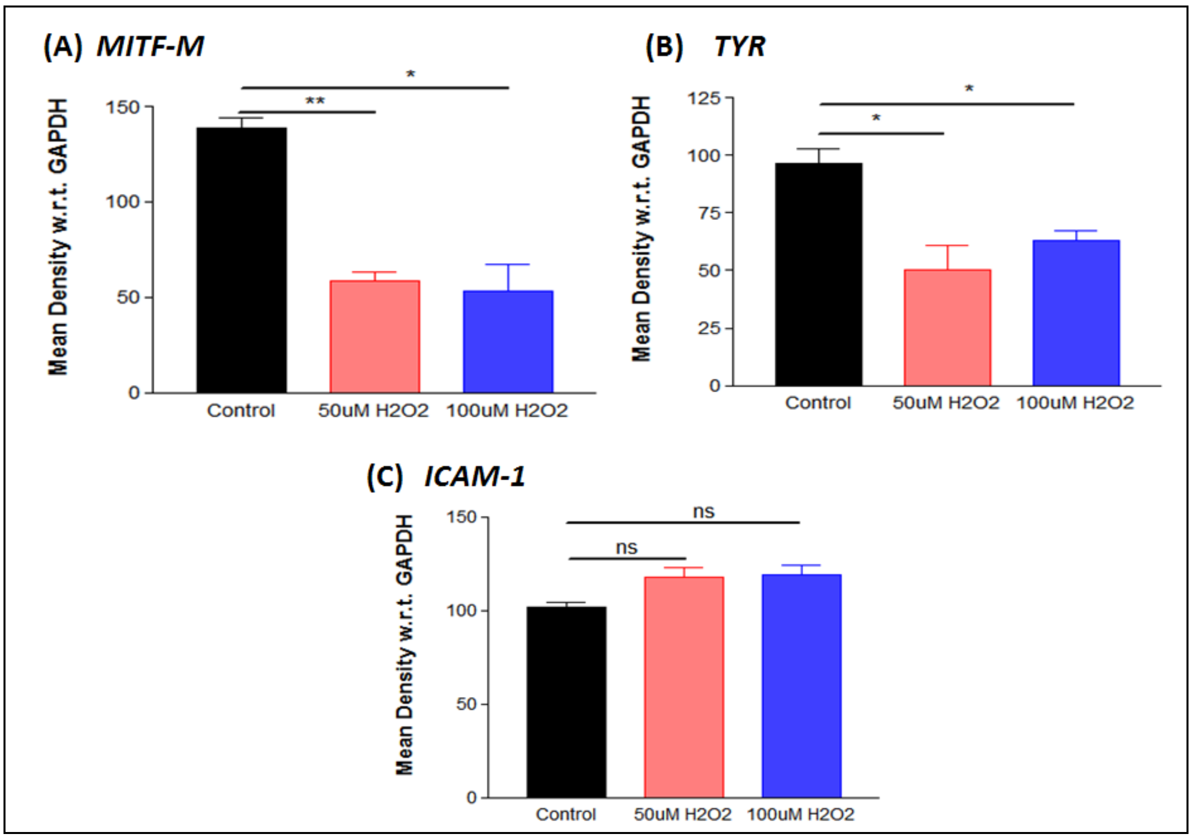
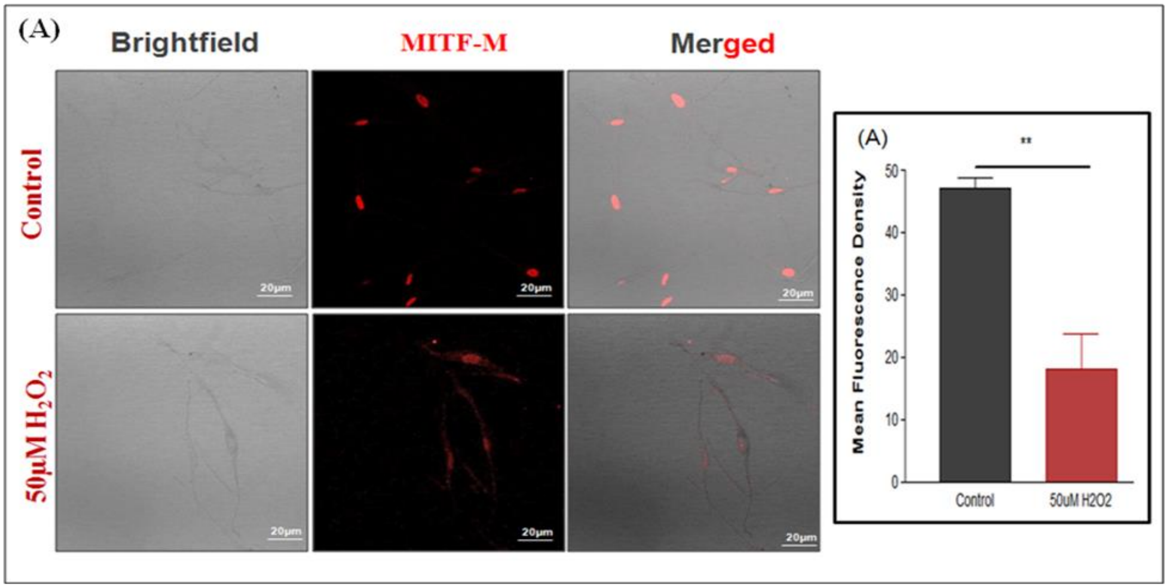


Figure 4: Effect of oxidative stress on *MITF-M, TYR* and *ICAM-1* mRNA expression: NHM were treated with 50 µM and 100 µM of H_2O_2 for 24 hrs (A) *MITF-M*

transcript levels were significantly reduced, *p*=0.0083 and *p*=0.0383 respectively. (B) *TYR* transcript levels were also significantly reduced, *p*=0.0439 and *p*=0.0109 respectively. (C) However, there was no significant difference in *ICAM-1* transcript levels, *p*=0.0772 and *p*=0.1325 respectively (n=3)



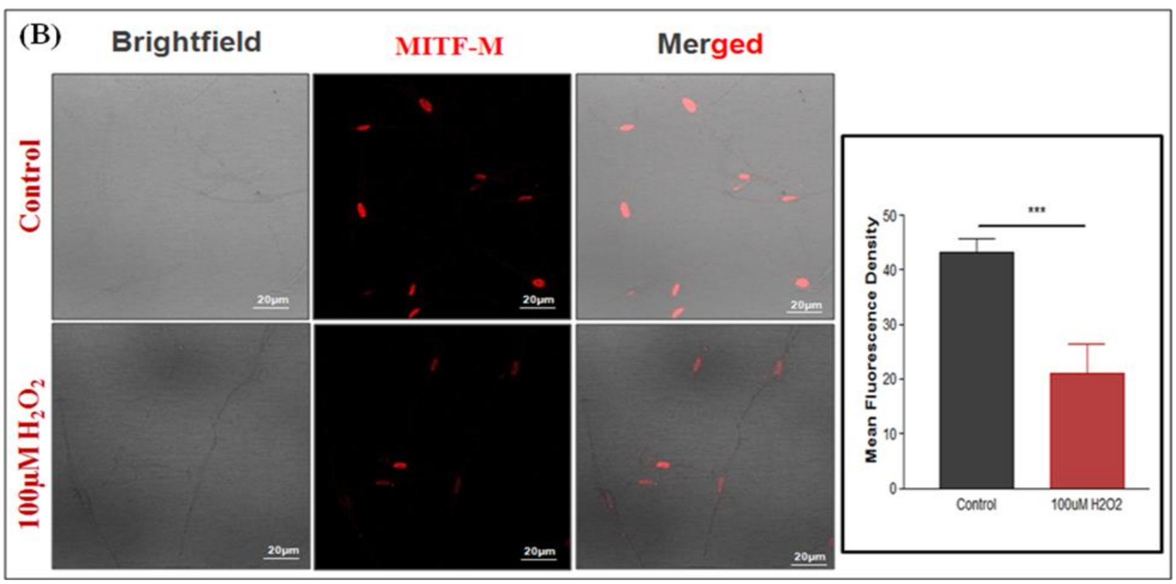


Figure 5: Dose dependent effect of H_2O_2 on MITF-M expression in NHM: Immunofluorescence analysis revealed significant decrease in MITF-M expression in NHM treated with (A) 50 μM and (B) 100 μM of H_2O_2 for 24 hrs (*p*=0.0024 and *p*=0.0001 respectively) (n=3; Magnification 630X: scale: 20μ).

6.4 DISCUSSION

Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis involving both genetic and non-genetic factors. The precise modus operandi for vitiligo pathogenesis has remained elusive (Laddha *et al*., 2013). PARP1 has been the most extensively studied member of PARP family. In response to DNA-strand breaks, it uses NAD^+ as a substrate to catalyze the covalent attachment of ADP-ribose units on the γ-carboxyl group of glutamate residues of acceptor proteins that are usually associated with DNA transactions, transmodification or on PARP1 itself (Hassa *et al*., 2008). PARP causes DNA damage-induced poly(ADPribosyl)ation of the N- and C-terminal tails of histone H1 and H2B and increases the access to breaks which in turn stimulates DNA repair signaling mechanism. Alteration of PARP1 activity may affect the development and functioning of cells through multiple processes. PARylation reaction is involved in several physiological processes such as differentiation, transcriptional regulation etc. However, the mechanism underlying the role of PARP1 in inflammatory response is still not clear. During inflammation, oxygen radicals are produced which induce massive DNA damage resulting in PARP1 over activation, leading to cell death by energy depletion. PARP1 is also found to modulate the transcription of inflammation linked genes. Thus, PARP1 regulates nuclear factor-κB (NF-κB)–dependent transcription and synthesis of inflammatory mediators, i.e., IL-1, IL-6, TNF-α, and inducible nitric oxide (NO) synthase (Oliver *et al*., 1999) which is a key element in the pathophysiology of inflammation and cell destruction. Various inflammatory cytokines, chemokines, necrotic cell products, bacteria and viruses stimulate NF-kB activation. Several investigators have proved the presence of oxidative stress in cultured melanocytes coupled with an increased susceptibility to pro-oxidants (Dell'Anna *et al*., 2007, Maresca *et al*., 1997; Boissy *et al*., 2004). *In vivo* oxidative stress has been attributed to accumulation of H_2O_2 in vitiligenous skin, which is associated with impaired catalase and glutathione peroxidase activities (Schallreuter *et al*., 1999). Also, millimolar levels of H_2O_2 lead to the inactivation of catalase due to substrate inhibition (Schallreuter *et al*., 1991); and glutathione peroxidase activities are found to be significantly decreased (Beazley *et al*., 1999, Agrawal *et al*., 2004). An alteration in melanocyte-specific proteins by the action of ROS results in the generation of neoantigens, autoimmunity and melanocytorrhagy leading to defective apoptosis. In the present study, H_2O_2 exposure caused a significant dose-dependent death of NHM. Recent findings suggest PARPs as potential chemotherapeutic targets (Leung *et al*., 2011). The first PARP inhibitors were nicotinamide analogues like benzamide, 3-aminobenzamide (Durkacz *et al*., 1980). Other popular PARP inhibitors include 4-aminol & naphthalimide, 6 (5N)- and 2-nitro-6(5W) phenanthridinones, and DHQ etc (Banasik *et al*., 1992). We have used DHQ as it has a very low IC_{50} of 0.39 µM toward PARP and a very high IC_{50} of 890 µM toward mono(ADP-ribosyl) transferase. DHQ is also the only inhibitor to produce 100% inhibition of PARP activity in *in vitro* at a 1mM concentration (Banasik *et al*., 1992). PJ34 is another potent novel PARP inhibitor that dose-dependently inhibits purified PARP enzyme in a cell-free assay with half maximal effective concentration EC50 value of 20 nM. Unlike other PARP inhibitors (such as 3-AB), PJ34 does not possess any antioxidant properties but exhibits 10,000 times greater PARP inhibition than 3-AB (EC50 = 200 µM) (Huang *et al*., 2008). PJ34 has been found to have neuro-protective effects and enhance the chemotherapeutic effects in several tumor types. Study results have shown that PJ34 inhibits peroxynitrite-induced cell necrosis with EC50 value of 20 nM and dose-dependently suppresses the growth of HepG2 cells (Galaleldin *et al*., 2001).

Our results showed that DHQ, a potent PARP inhibitor did not show any significant effect on NHM viability and morphology up to 24 hrs of treatment. On the contrary PJ-34, another PARP inhibitor (10 µM) independent of DNA damage repair induces cell death in M14 melanoma cells (Chevanne *et al*., 2010). We have studied DNA damage-dependent role of PARP triggered by ROS in NHM. Pre-treatment with PARP inhibitor, DHQ showed a marked rescue from H_2O_2 induced melanocyte death, suggesting a significant role of PARP1 in the oxidative stress induced cell death. PARylation pattern was also studied in H_2O_2 mediated oxidative stress. We found significant decrease in H_2O_2 mediated PARylation and PARP activation upon pre-treatment with DHQ (100 µM). Previous reports have also suggested rescue of chondrocytes exposed to increased H_2O_2 concentrations (Shin *et al*., 2012). DHQ has also been shown to inhibit PARP activity and peroxynitrite-induced cell necrosis in mouse thymocytes (Garcia *et al*., 2001). DHQ provided progenitors and mature oligodendrocytes with protection against H_2O_2 toxicity (Gabriela *et al*., 2004). Similar to observations by Liu *et al.*, (2009) and Dell'Anna *et al.*, (2006), our results demonstrated significant decrease in *MITF-M* expression at transcript and protein levels under oxidative stress conditions. Reduced expression of *TYR* was also observed by Jiménez-Cervantes *et al*., (2001). We did not find significant difference in *ICAM1* expression. However, increased *ICAM1* expression was reported in vitiligo patients (Dwivedi *et al.*¸ 2013; Al Badri 1993).

In conclusion, our results suggest that PARP-1 inhibitor, DHQ attenuates H_2O_2 induced NHM death and therefore, it could be used as a potential agent in vitiligo therapeutics.

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