Original Research Article

International Journal of Research and Review
Vol.8; Issue: 2; February 2021
Website: www.ijrrjournal.com
E-ISSN: 2349-9788; P-ISSN: 2454-2237

An *in Vitro* Study Elucidating the Effect of Oxidative Stress on Melanocytes

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ABSTRACT

Oxidative stress plays a major role in melanocyte destruction in vitiligo; however the exact mechanism responsible for melanocyte death remains uncertain. We aimed to examine the effect of oxidative stress on melanocyte viability by MTT assay and expression of antioxidant genes (*CAT, GPX1, G6PD* and *PRDX3*), stress related genes (*HSP60, HSP70, SERP1, SIRT1* and *POLH*) and melanocyte specific genes (*MITF, TYR, TYRP1, TRPM1, EDN1, EZR* and *LAMP1*) by real-time PCR upon exposing the normal human melanocytes (NHM), immortalized melanocytes derived from healthy human (PIG1) and from vitiligo patient (PIG3V) to cumene hydroperoxide (CHP). The transcript levels of selected genes were estimated by using real-time PCR. The NHM, PIG1 and PIG3V melanocytes showed significant decrease in viability under CHP (10-100μM) induced oxidative stress. PIG3V displayed significantly increased expression of *PRDX3, HSP70, SERP1, POLH* as well as decreased expression of *CAT, MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* under CHP (10 & 20μM) treatment, as compared to NHM and/or PIG1 melanocytes. These results suggest that vitiligo melanocytes are more sensitive to CHP induced oxidative stress, as compared to normal melanocytes. The present study demonstrates that vitiligo may result from an insufficient response of melanocytes to oxidative stress induced by high H_2O_2 levels.

Key words: Vitiligo; melanocyte; PIG1; PIG3V; oxidative stress; cumene hydroperoxide (CHP).

1. INTRODUCTION

The skin consists of the epidermis, the dermis, and a basement membrane which contains melanocytes originating from neural crest cells (Fuchs and Raghavan 2002; Proksch *et al.* 2008). Melanocytes, because of their vicinity, are liable to be attacked by several of exogenous chemicals (Bickers and Athar 2006). These environmental toxicants or their metabolites may produce various reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) (Bogeski *et al.* 2012). Uncontrolled release of ROS drives the induction of oxidative stress that can cause the destruction of melanocytes leading to vitiligo (Briganti and Picardo 2003; Spritz 2008). The presence of oxidative stress in both skin and blood of vitiligo patients has been well established (Beazley *et al.* 1999; Schallreuter *et al.* 1999). H_2O_2 amasses in the epidermis of patients, concomitant with reduced levels of catalase (Schallreuter *et al.* 1999). Altered antioxidant levels, including catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPX), glucose 6-phosphate dehydrogenase (G6PD) and superoxide dismutase (SOD), and lipid peroxidation (LPO), was seen in vitiligo patients (Passi *et al.* 1998; Agrawal *et al.* 2004; Koca *et al.* 2004; Shajil and Begum 2006; Em *et al.* 2007; Laddha *et al.* 2013, 2014; Mansuri *et al.* 2016a) resulting into oxidative damage to melanocytes. These conditions may induce expression of stress proteins including heat shock protein

70 (HSP70) and will enhance the activity of anti-oxidant enzymes to protect the cell (Calabrese *et al.* 2001; Renis *et al.* 2003).

Elevated H_2O_2 levels can alter calcium homeostasis (Schallreuter *et al.* 2007). The transient receptor potential cation channel, subfamily M, member 1 (TRPM1) is a constitutively active Ca^{2+} channel, which is expressed in melanocytes and its activity is critical for melanocyte homeostasis(Hunter *et al.* 1998; Gaur *et al.* 2007; Devi *et al.* 2009). Moreover, it has been stated that the *TRPM1* expression is microphthalmia-associated transcription factor (MITF) reliant, which is considered as a key melanocyte regulator, controlling the expression of genes involved in melanogenesis including tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1) (Fang and Setaluri 1999; Widlund and Fisher 2003; Levy *et al.* 2010). The expression of *MITF* leads to a reduced oxidative stress response, suggesting its role in melanocyte stress response mechanism (Jiménez-Cervantes *et al.* 2001). However, the mechanisms underlying the aberrant responses induced by ROS-mediated melanocyte loss are not completely understood. Hence, we aimed to evaluate melanocyte viability, and to investigate the transcript expression levels of anti-oxidant genes (*CAT, GPX1, G6PD* and *PRDX3*), stress related genes (*HSP60, HSP70, SERP1, SIRT1* and *POLH*) and melanocyte specific genes (*MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1*) in NHM, PIG1 and PIG3V cells under CHP induced oxidative stress.

2. MATERIALS AND METHODS

2.1. Ethics statement

The scheme of the present study was permitted by Institutional Ethics Committee for Human Research (IECHR) of Faculty of Science, The Maharaja Sayajjirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all the participants for collecting the skin biopsies for NHM and written consent was obtained.

2.2. Culture establishment of primary normal human melanocytes (NHM)

Melanocytes were isolated from human skin biopsy samples and cultured successfully using the standard protocol with slight modifications (Im *et al.* 1993; Czajkowski *et al.* 2007). Melanocytes were used for experiments in the fifth or sixth passage.

2.3. Human Melanocyte Cell lines

Immortalized human melanocyte cell lines PIG1 (derived from healthy individual) and PIG3V (derived from vitiligo patient) were received from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois and cultured as described by Le Poole (Le Poole and Boissy 1997; Le Poole *et al.* 2000).

2.4. MTT assay

The cell viability was monitored using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium salts]. NHM/ PIG1/ PIG3V cells were seeded in 96-well plate at a density of about 5000 cells in each well. Cells were treated with cumene hydroperoxide (CHP) (Sigma Aldrich, USA) in a dose dependent manner (10, 20, 40, 60, 80 & 100 μM). After 24 hrs of treatment, MTT Assay (Molecular probes® by Life Technologies™, China) was performed as per the manufacturer's instructions.

2.5. RNA isolation and cDNA synthesis

Total RNA from NHM/ PIG1/ PIG3V cells was extracted using RNA isolation kit (Ambion®, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA synthesis was performed using Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the Mastercycler Gradient PCR (Eppendorf, Germany).

2.6. Gene expression analysis

The transcript levels of anti-oxidant, stress related and melanocyte specific genes in CHP treated and untreated cells were

estimated by real-time PCR using SYBR green method and gene specific primers (Eurofins, Bangalore, India) as shown in Table S1. *GAPDH* was considered as a housekeeping gene. Real-time PCR was performed in duplicate using LightCycler®480 SYBR Green I Master following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany).

Table S1. Details of primers used for mRNA expression of the candidate genes.

Gene	Forward/ reverse primer	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Amplicon size (bp)
EDN1	FP	ACTTCTGCCACCTGGACATCA	63	91
	RP	TCCAAGGCTCTCTTGGACCTAG		
EZR	FP	TCCCTC AAAGAG TGATGG ACCAG	65	100
	RP	TTA TCT TTG AGC ATC CCA CGG TG		
LAMP1	FP	GCGAGCTCCAAAGAAATCAA	63	95
	RP	TGGACCTGGGTGCCACTAA		
POLH	FP	ATCATGGAAGGGTGGTGGAAT	63	167
	RP	TGGCTTCCCGGTACTTGG		
SIRT1	FP	ACG CTG GAA CAG GTT GCG G	64	168
	RP	AAG CGG TTC ATC AGC TGG GC		
SERP1	FP	TCGCCAAGACCTCGAGAAATG	62	101
	RP	CTGGAAAATTGCAGAACCACAGAC		
TYR	FP	AGCACCCCACAAATCCTAACTTAC	63	92
	RP	ATGGCTGTTGTACTCCTCCAATC		
TRPM1	FP	ACTCTAACAGGTGTTGCTGTGG	62	153
	RP	CTGTTGGGTAGCTCTGGGTG		
HSP60	FP	CTGGTGGTGCAGTGTTTGG	62	269
	RP	TGTCCCACCAACCTTCAGC		
HSP70	FP	TGAAGAAGGGTCAAGTGACTGTG	62	162
	RP	ACTGAAAACTGAGCTATAGCAGG		
PRDX3	FP	TTCAGCACCAGTTCCTCATG	60	168
	RP	AGGACACACACAAAGGTGAAATCC		
TYRP1	FP	TTT GTA ACA GCA CCG AGG ATG	62	192
	RP	TGG GGT CAC TGT AAC CTT CCA C		
G6PD	FP	TGAGCCAGATAGGCTGGAA	63	225
	RP	TAACGCAGGCGATGTTGTC		
MITF	FP	CAAATGATCCAGACATGCGCTGG	61	180
	RP	CTCGAGCCTGCATTTCAAGTTCC		
CAT	FP	TAAGACTGACCAGGGCATC	63	201
	RP	CAAACCTTGGTGAGATCGAA		
GPX1	FP	GTTTGGGCATCAGGAGAACGCC	64	147
	RP	AGGAAGGCGAAGAGAGGGTGC		
GAPDH	FP	ATCCCATCACCATCTTCCAGGA	65	122
	RP	CAAATGAGCCCCAGCCTTCT		

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

2.7. Statistical analysis

All the experiments were performed at least three times in triplicates independently on different days using different batches of cells, and data are presented as the mean ± SEM. To evaluate the MTT or gene expression results, the absorbance or ΔCt values respectively, were compared between different groups and analysis was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine the statistical significance of data. $p < 0.05$ was considered statistically significant. Fold change in mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method.

3. RESULTS

3.1. Dose dependent effect of CHP on melanocyte viability

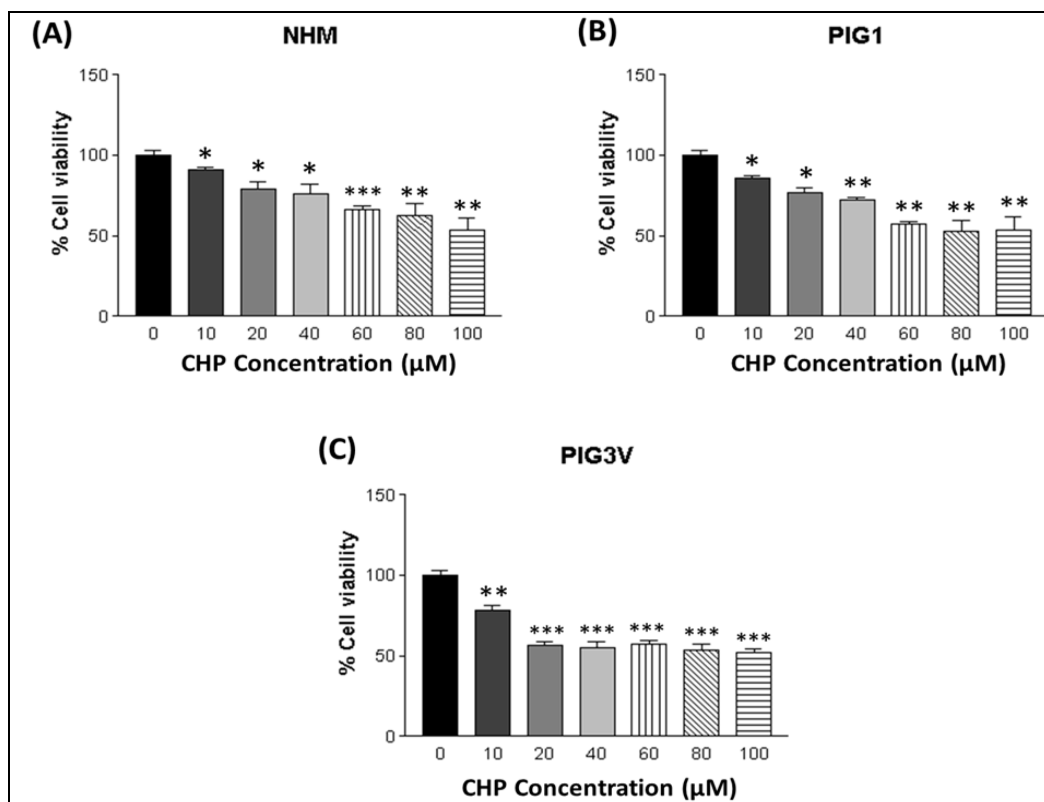

Figure 1. Dose dependent effect of CHP on melanocyte viability: (A) NHM, (B) PIG1 and (C) PIG3V cells showed significantly decreased viability upon 10, 20, 40, 60, 80 and 100 µM CHP treatments for 24 hrs as compared to untreated cells (n=3).

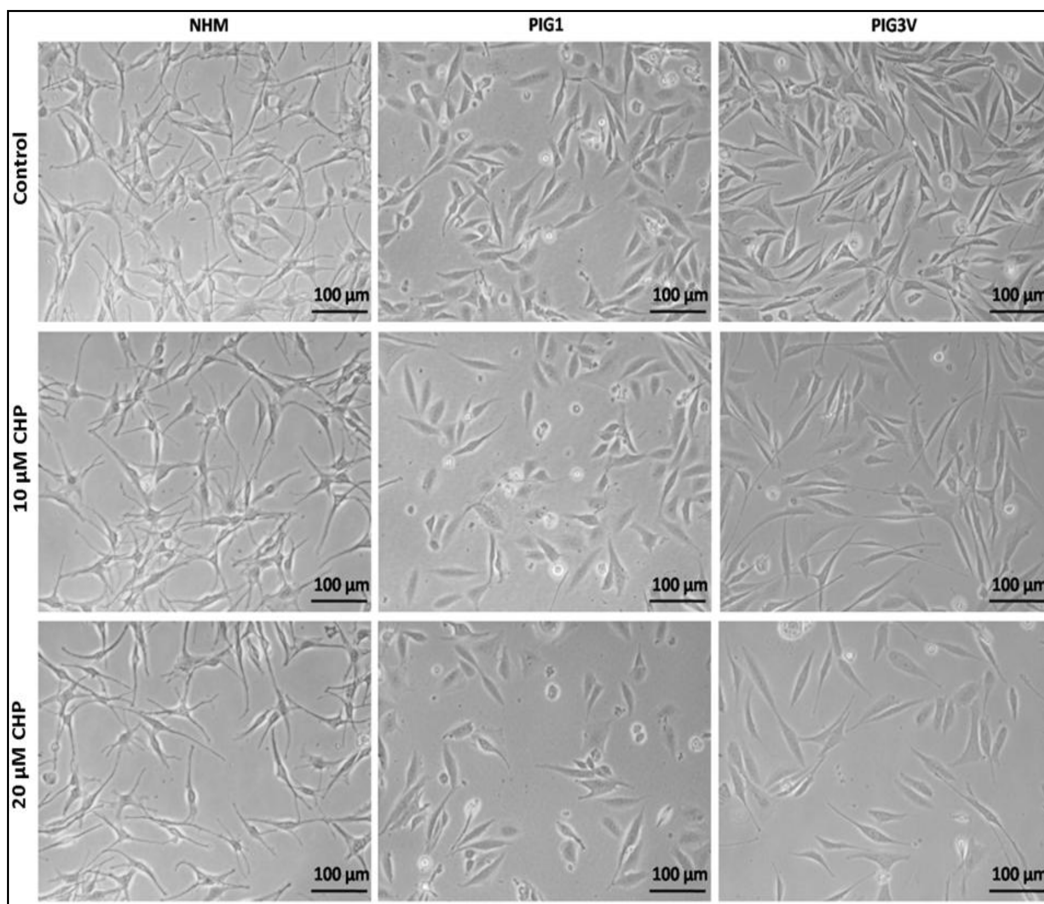

Figure 2. Effect of CHP on melanocytes: NHM, PIG1 and PIG3V cells showed significant decrease in viability upon 10 and 20 µM CHP treatments for 24 hrs as compared to untreated cells.

Normal human melanocytes (NHM)/ immortalized normal human melanocytes (PIG1) and immortalized melanocytes from vitiligo patient (PIG3V) were treated in a dose dependent manner with CHP (10-100µM) and observed after 24 hrs for viability. NHM showed significant decrease in viability (mean ± SEM) upon: 90.94 ± 1.50% at 10µM (p=0.049), 79.08 ± 4.37% at 20µM (p=0.016), 76.40 ± 5.81% at 40µM (p=0.022), 66.10 ± 2.48% at 60µM (p=0.0009), 62.96 ± 6.95% at 80µM (p=0.008) and 53.83 ± 7.09% at 100µM (p=0.004) CHP treatment for 24 hrs as compared to untreated NHM (Figure 1A). PIG1 showed significant decrease in viability (mean ± SEM) upon: 85.77 ± 1.60% at 10µM (p=0.036), 76.78 ± 3.43% at 20µM (p=0.014), 72.17 ± 1.90% at 40µM (p=0.006), 57.71 ± 1.03% at 60µM (p=0.002), 52.74 ± 6.93% at 80µM (p=0.005) and 53.60 ± 8.23% at 100µM (p=0.008) CHP treatment for 24 hrs as compared to untreated cells (Figure 1B). PIG3V also showed significant decrease in viability (mean ± SEM) upon: 78.16 ± 3.27% at 10µM (p=0.008), 56.63 ± 2.32% at 20µM (p=0.0003), 55.44 ± 3.57% at 40µM (p=0.0006), 57.23 ± 2.54% at 60µM (p=0.0004), 53.80 ± 3.35% at 80µM (p=0.0005) and 47.71 ± 3.54% at 100µM (p=0.0002) CHP treatment for 24 hrs as compared to untreated cells (Figure 1C). NHM and PIG1 cells showed similar susceptibility to H_2O_2 at all concentrations, whereas PIG3V cells showed more vulnerability to H_2O_2 upto 20 µM and exhibited around 50% cell death at all higher concentrations (Figure 1 & 2)

3.2. Gene expression profile of Melanocytes under normal condition

Expression of anti-oxidant genes *CAT, GPX1, G6PD* and *PRDX3;* stress related genes *HSP60, HSP70, SERP1, SIRT1* and *POLH;* and melanocyte specific genes *MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* in untreated NHM, PIG1 and PIG3V were monitored (Figure 3). However, no significant difference was observed in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG1 (p=0.976, p=0.7458, p=0.128, p=0.983 respectively; Figure 2). Further, no difference was observed in the expression of *CAT, GPX1, G6PD* and *PRDX3* between NHM and PIG3V (p=0.318, p=0.867, p=0.714 and p=0.664 respectively). Also, no significant difference in the expression of *CAT, GPX1, G6PD* and *PRDX3* was observed between PIG1 and PIG3V (p=0.217, p=0.883, p=0.070 and p=0.666 respectively). Significantly higher expression of *HSP70* and *SERP1* was observed in PIG3V cells as compared to PIG1 (p=0.015 and p=0.049 respectively; Figure 3). However, there was no difference observed for *HSP70* and *SERP1* expression between NHM and PIG3V (p=0.095 and p=0.447 respectively). Also, significant difference was not observed in the expression of *HSP60, SIRT1* and *POLH* between NHM and PIG3V (p=0.914, p=0.312 and p=0.287 respectively). No significant difference was observed in *HSP60, HSP70, SERP1, SIRT1* and *POLH* expression was observed between PIG1 and NHM (p=0.400, p=0.091, p=0.054, p=0.875 and p=0.217). Moreover, expression of *HSP60, SIRT1* and *POLH* was not significantly different between PIG1 and PIG3V cells (p=0.355, p=0.355, p=0.951). *TYR, TRPM1* and *EZR* expression was significantly decreased in PIG3V as compared to NHM (p=0.031, p=0.012 and p=0.022 respectively). Though, no change was observed in *MITF, TYRP1, EDN1* and *LAMP1* expression in PIG3V and NHM (p=0.155, p=0.134, p=0.647 and p=0.151 respectively). Further, no difference was observed in the expression of *MITF, TYRP1, TRPM1, EDN1, EZR* and *LAMP1* between PIG1 and NHM (p=0.197, p=0.405, p=0.986, p=0.968, p=0.331, p=0.692 and p=0.685 respectively). Also, there was no difference observed in the expression of *MITF, TYRP1, TRPM1, EDN1, EZR* and *LAMP1* between PIG3V and PIG1 (p=0.725, p=0.179, p=0.216, p=0.022,

p=0.564, *p*=0.125 and *p*=0.273 respectively).

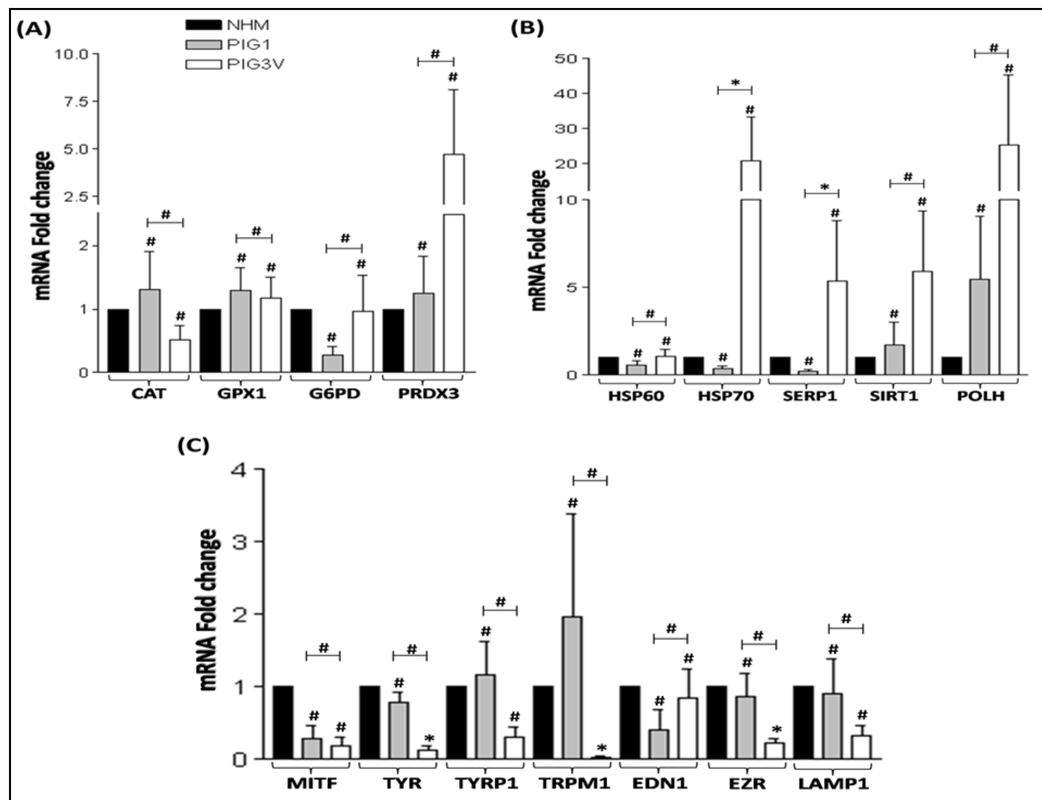

Figure 3. Gene expression profiles of NHM, PIG1 and PIG3V cells under normal condition. (A) Anti-oxidant genes: There was no difference in expression of *CAT, GPX1, G6PD* and *PRDX3* among melanocytes. **(B) Stress related genes:** PIG3V showed higher expression of *HSP70* and *SERP1* as compared to PIG1. There was no difference in expression of *HSP60, SIRT1* and *POLH* among melanocytes. **(C)Melanocyte specific genes:** PIG3V showed lower expression of *TYR, TRPM1* and *EZR* as compared to NHM. There was no difference in expression of *MITF, TYRP1, EDN1* and *LAMP1* among melanocytes. [$^{*}p<0.05$; $^{\#}p>0.05$ or non-significant]

3.3. Gene expression profile of melanocytes under CHP induced oxidative stress

NHM, PIG1 and PIG3V were treated with CHP (10µM & 20µM) (Figure 2) and transcript levels of anti-oxidant genes, stress related genes and genes related to melanocytes were investigated after 24 hrs.

3.3.1. mRNA expression levels at 10µM CHP

PIG3V cells indicated markedly reduced *CAT* expression as compared to NHM (*p*=0.012) and increased *PRDX3* expression as compared to PIG1 (*p*=0.039). Although, there was no difference in *CAT, GPX1, G6PD* and *PRDX3* expression among NHM and PIG1 cells (*p*=0.071, *p*=0.244, *p*=0.062 and *p*=0.808 respectively; Figure 4A). No significant difference in G*PX1, G6PD* and *PRDX3* expression was observed between PIG3V and NHM (*p*=0.703, *p*=0.923 and *p*=0.427 respectively). Also, no significant change in *CAT, GPX1* and *G6PD* expression between PIG1 and PIG3V (*p*=0.654, *p*=0.750 and *p*=0.128).

PIG3V exhibited a significantly increase in *HSP70* expression as compared to NHM (*p*=0.023), and higher expression of *HSP70, SERP1* and *POLH* in comparison to PIG1 (*p*=0.008, *p*=0.043 and *p*=0.043 respectively; Figure 4B). However, there was no difference in *HSP60, HSP70, SERP1, SIRT1* and *POLH* expression between PIG1 and NHM (*p*=0.579, *p*=0.636, *p*=0.124, *p*=0.534 and *p*=0.114 respectively). In addition, no difference in *HSP60, SERP1, SIRT1* and *POLH* expression was between PIG3V and NHM (*p*=0.969, *p*=0.476, *p*=0.352 and *p*=0.356 respectively). Moreover, the results suggested no difference in *HSP60* and *SIRT1* expression between PIG1 and PIG3V

(p=0.361, and p=0.888). The PIG3V shown significantly decreased *MITF, TYR, TRPM1, EDN1* and *EZR* expression as compared to NHM (p=0.017, p=0.015, p=0.0003, p=0.014 and p=0.037 respectively; Figure 4C). Also, we observed that *MITF, TYR, TRPM1, EZR* and *LAMP1* expression was significantly decreased in PIG3V as compared to PIG1 upon CHP treatment (p=0.039, p=0.007, p<0.0001, p=0.009 and p=0.008 respectively). However, there was no difference in *MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* expression between PIG1 and NHM (p=0.638, p=0.771, p=0.670, p=0.139, p=0.433, p=0.524 and p=0.661 respectively). Further, no difference was observed in *TYRP1* and *LAMP1* expression between PIG3V and NHM (p=0.772 and p=0.145 respectively). Also, there was no difference observed in *TYRP1* and *EDN1* expression between PIG3V and PIG1 (p=0.589 and p=0.915 respectively).

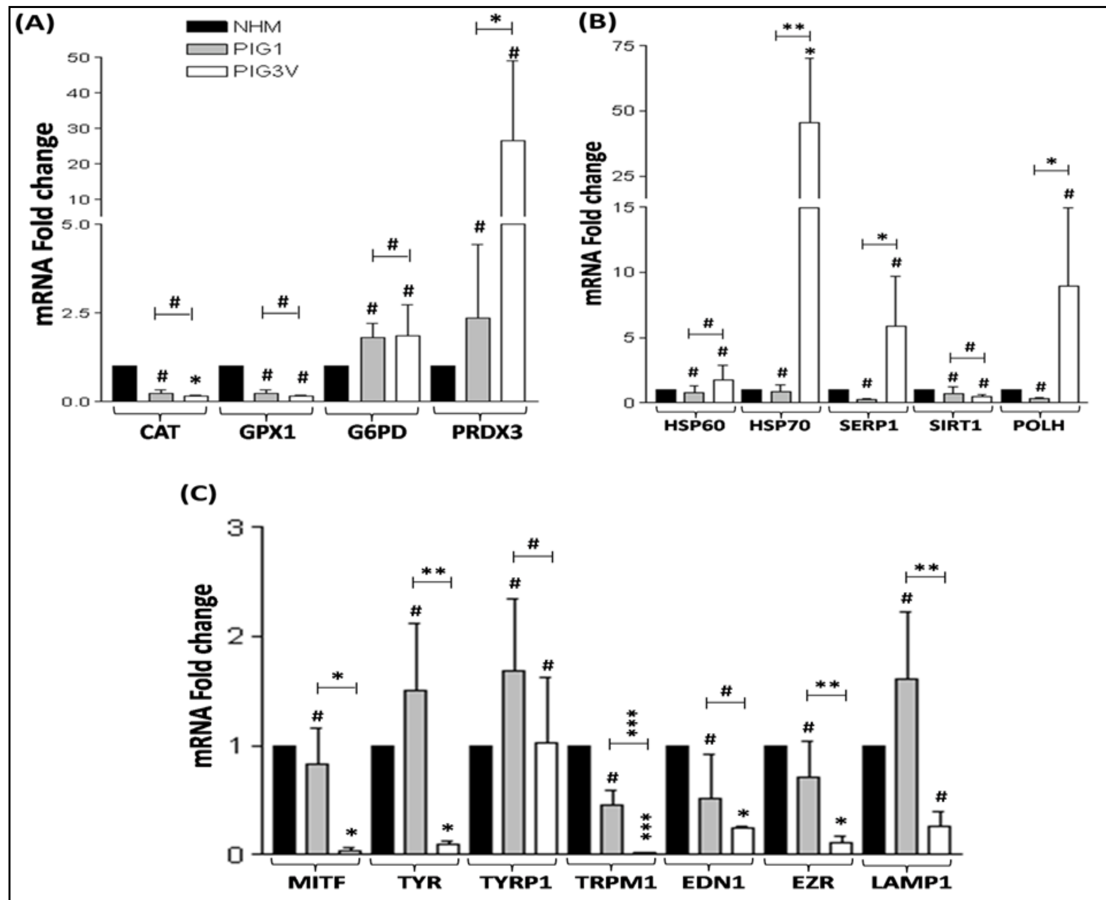

Figure 4. Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 hrs of 10µM CHP treatment. (A) Expression profile of anti-oxidant genes (B) Expression profile of stress related genes (C) Expression profile of melanocyte specific genes. [*p<0.05; **p<0.01; ***p<0.001; #p>0.05 or non-significant]

3.3.2. mRNA expression levels at 20µM CHP

PIG3V and PIG1 showed significantly decreased *CAT* expression as compared to NHM (p=0.019 and p=0.033 respectively; Figure 5A). PIG3V showed an increased *PRDX3* expression in comparison to NHM and PIG1 (p=0.005 and p=0.003 respectively). No significant difference in *GPX1, G6PD* and *PRDX3* expression between NHM and PIG1 was observed (p=0.088, p=0.078 and p=0.683 respectively). Further, no difference was observed in *GPX1* and *G6PD* expression between PIG3V and NHM (p=0.127 and p=0.746 respectively). Also, we did not observe any significant difference in *CAT, GPX1* and *G6PD* expression between PIG1 and PIG3V (p=0.159, p=0.871 and p=0.488 respectively). *HSP70* expression was

significantly increased in PIG3V as compared to NHM (p=0.047) and augmented *HSP70, SERP1* and *POLH* expression in comparison to PIG1 (p=0.001, p=0.044 and p=0.043; Figure 5B). PIG1 also showed significantly increased *POLH* expression as compared to NHM (p=0.039). However, there was no difference in *HSP60, HSP70, SERP1* and *SIRT1* expression between PIG1 and NHM (p=0.129, p=0.217, p=0.067 and p=0.905 respectively). No difference was observed in *HSP60* and *SIRT1* expression between PIG3V and NHM (p=0.395 and p=0.154 respectively). Further, no difference was observed in *HSP60* and *SIRT1* expression between PIG1 and PIG3V cells (p=0.085, and p=0.505 respectively). The PIG3V cells indicated a significant downregulation of *MITF, TYR, TRPM1* and *EZR* expression as compared to NHM (p=0.021, p=0.0004, p=0.032 and p=0.009 respectively; Figure 5C). Moreover, PIG3V indicated downregulation of *TYR, TYRP1, TRPM1* and *EZR* expression as compared to PIG1 (p=0.012, p=0.044, p=0.027 and p=0.021 respectively). However, there was no difference in *MITF, TYR, TYRP1, TRPM1, EDN1* and *LAMP1* expression between PIG1 and NHM (p=0.263, p=0.081, p=0.815, p=0.056, p=0.916 and p=0.613 respectively). Additionally, no difference in *TYRP1, EDN1* and *LAMP1* expression was observed between PIG3V and NHM (p=0.176, p=0.210 and p=0.139 respectively). Also, there was no difference in *MITF, EDN1* and *LAMP1* expression between PIG3V and PIG1 (p=0.158, p=0.482 and p=0.325 respectively).

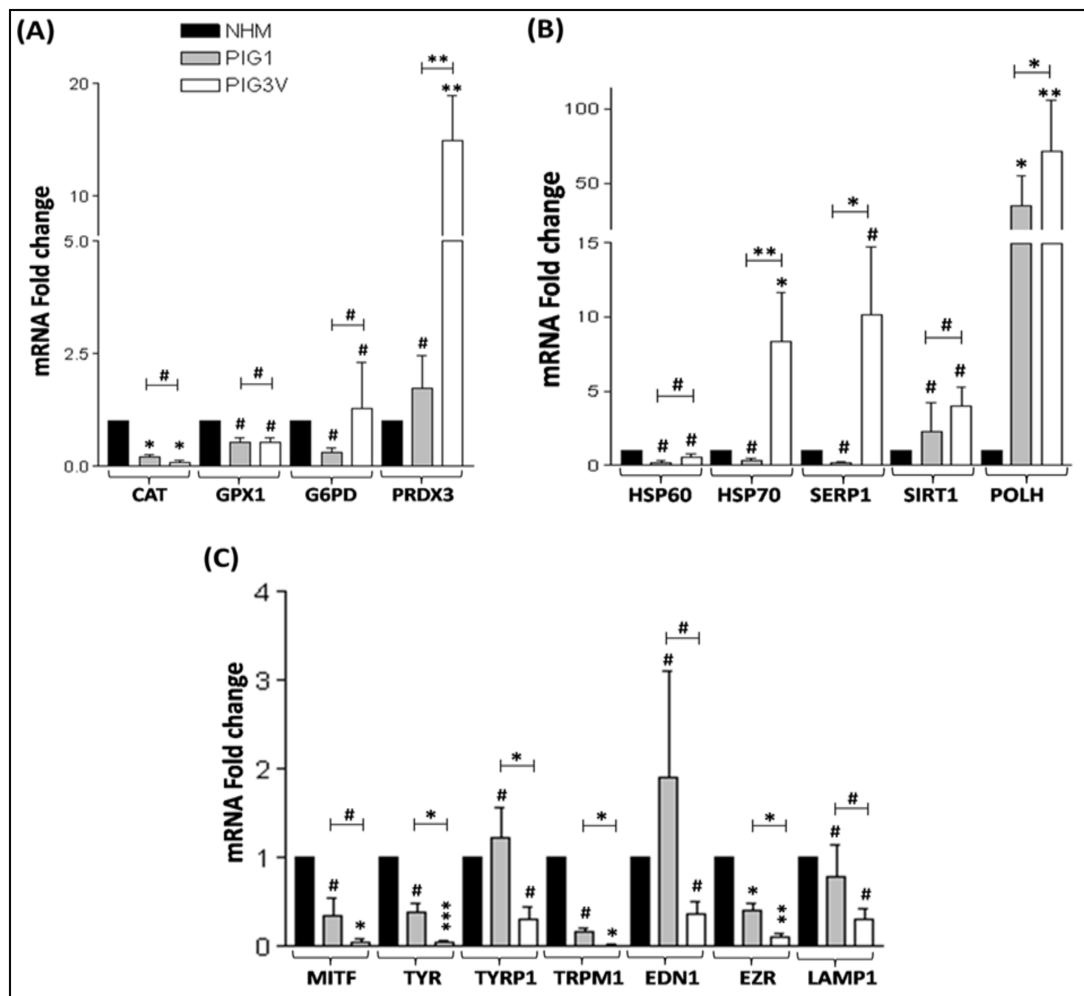

Figure 5. Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 hrs of 20µM CHP treatment. (A) Expression profile of anti-oxidant genes (B) Expression profile of stress related genes (C) Expression profile of melanocyte specific genes. [$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; $^{\#}p>0.05$ or non-significant]

4. DISCUSSION

Hampered (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin ($6BH_4$) regulation; impaired catecholamine production in addition to an augmented monoamine oxidase A (MOA) and low GPX activity are the among the major sources for epidermal H_2O_2 generation in vitiligo (Schallreuter *et al.* 1999). Over production of H_2O_2 may cause catalase inactivation and vacuolation in the epidermal melanocytes and keratinocytes. Interestingly, vitiligo melanocytes exhibited 'vacuolation' *in vitro* in which was reversible upon addition of catalase (Schallreuter *et al.* 1999).

Earlier, Maresca *et al.* have demonstrated that vitiligo melanocytes were susceptible to the toxic effect of CHP (Maresca *et al.* 1997). Similarly, in the present study PIG3V were found to be more sensitive to CHP in contrast to PIG1 and NHM (Figure 1). Further, PIG3V exhibited downregulation of *CAT* expression in comparison to NHM upon CHP exposure (Figures 2 and 3), which is in accordance with previous study (Maresca *et al.* 1997). Peroxiredoxins (PRDXs) act as free radical scavenger and are also involved in the degradation of H_2O_2(Gourlay *et al.* 2003; Wood *et al.* 2003; Sue *et al.* 2005). Earlier, we have reported the up-regulated *PRDX3* in skin and blood of vitiligo patients (Mansuri *et al.* 2016b). *PRDX3* was found to elevated in PIG3V melanocytes as compared to NHM and/ PIG1 melanocytes (Figures 2 and 3), suggesting its protective role in vitiligo melanocytes.

Consequently, local and systemic high levels of H_2O_2 are able to alter the calcium homeostasis in melanocytes (Schallreuter *et al.* 2007). Schallreuter *et al.* have shown that cells from the lesional skin showed decreased Ca^{2+} uptake (Schallreuter-Wood *et al.* 1996). The present study suggests that *TRPM1* expression is decreased in PIG3V melanocytes (Figure 3). Moreover, PIG3V melanocytes showed significantly decreased expression of *TRPM1* in response to CHP as compared to NHM and/ PIG1 (Figures 3 and 4), indicating its essential role in vitiligo pathogenesis.

Differential expression of stress proteins HSP60 and HSP70 is reported in the skin of vitiligo patients including our recent study (Thörneby-Andersson *et al.* 2000; Mosenson *et al.* 2012; Mansuri *et al.* 2016b). In the present study, PIG3V showed higher expression of *HSP70* in response to CHP induced oxidative stress (Figures 3 and 4) which is in accordance with the previous study where, exposure to 4-tertiary butyl phenol (4-TBP) enhanced the expression of HSP70 in melanocytes (Kroll *et al.* 2005). 4-TBP is reported to induce oxidative stress (O'Brien 1991; Thörneby-Andersson *et al.* 2000). Also, PIG3V showed a tendency to be more sensitive to 4-TBP as compared to PIG1 (Kroll *et al.* 2005). Recently, Sastry *et al.*, have reported significant upregulation of *HSP70* expression in H_2O_2 treated PIG1 cells (Sastry *et al.* 2019). Asea et al., reported that HSP70 induced monocytes/macrophages showed secretion of cytokines such as IL-1, IL-6, and TNF-α (Asea *et al.* 2000). Also it has been shown that melanocytes can generate cytokines as well and elevated levels of TNF-α are reported (Krüger-Krasagakes *et al.* 1995)

SERP1 stabilizes membrane proteins during stress and facilitates subsequent glycosylation, which protects unfolded target proteins against degradation during ER stress (Yamaguchi *et al.* 1999). Whereas, EZR acts as a linker between the plasma membrane and cytoskeleton; and interacts with intercellular adhesion molecules 1 and 2 (Vaheri *et al.* 1997). Defective cell proliferation and adhesion mediated events were observed in mutant EZR (Y145F) expressing epithelial cells (Srivastava *et al.* 2005). *SERP1* and *EZR* were found to be down-regulated in the lesional and non-lesional skin and blood of patients (Mansuri *et al.* 2016b). In contrast, PIG3V melanocytes exhibit higher levels of *SERP1* and lower levels of *EZR* as compared to PIG1 and NHM respectively (Figure 3), indicating accumulation of unfolded proteins in vitiligo melanocytes.

Additionally, PIG3V melanocytes showed increased *SERP1* expression as compared to NHM and/ PIG1 cells (Figures 3 and 4). However, *EZR* was down-regulated in PIG3V melanocytes in response to CHP as compared to NHM and/ PIG1 cells (Figure 4 & 5). Previously, we have proposed that ER stress might be playing an important role in connecting oxidative stress and autoimmunity in vitiligo (Shoab Mansuri *et al.* 2014). This is further supported by decreased expression of *EZR* and increased expression of *SERP1* in vitiligo melanocytes.

Endothelin 1 (EDN1) is a paracrine growth factor synthesized by numerous cell types including keratinocytes, which interacts synergistically with α-MSH and basic fibroblast growth factor that together affect melanocyte proliferation, migration, tyrosinase activity, melanogenesis, and dendrite formation (Hara *et al.* 1995; Tada *et al.* 1998). EDN1 also increases the expression and phosphorylation of MITF (Kadekaro *et al.* 2005). Manga *et al*. (Manga *et al.* 2006) have demonstrated that EDN1 caused an increased melanocyte susceptibility to 4-TBP and that MITF expression is reliant on the redox condition of cells (Jiménez-Cervantes *et al.* 2001). Previously, we showed that *EDN1* was down-regulated in lesional skin and blood of vitiligo patients (Mansuri *et al.* 2016b). The present study showed significantly decreased expression of *EDN1* in PIG3V as compared to NHM in response to 10µM CHP (Figure 4), suggesting an important role for EDN1 in the regulation of human melanocytes. Pathways involved in MITF regulation were found to be defective in vitiligo (Kitamura *et al.* 2004). Reduced MITF levels might lead to decreased expression of *TYRP1* and related genes resulting in activation of apoptosis during oxidative stress (Manga *et al.* 2006). *TYR* and *TYRP1* are expressed in melanocytes and mainly localized in melanosomes where they play key roles in promoting melanogenesis (Sturm and Duffy 2012). LAMP1 is a vesicular membrane glycoprotein of melanocytes (Zhou *et al.* 1993). TYR, TYRP1 and LAMP1 are expressed as a multi-protein complex and function together by stabilizing the enzyme-protein complex within the melanosome and prevent the premature death of melanocytes due to tyrosinase-mediated cytotoxicity (Ghanem and Fabrice 2011). Jimbow *et al*. have indicated that higher susceptibility of vitiligo melanocytes is concordant to the higher sensitivity for oxidative stress (ultraviolet B) (Jimbow *et al.* 2001), which may arise from abnormal synthesis, altered folding and maturation of nascent TYRP1 polypeptides along with decreased expression of *TYRP1* in vitiligo melanocytes was demonstrated (Luo *et al.* 1994). MITF stimulates melanin synthesis by regulating expression of melanogenic enzymes (TYR and TYRP1) and reduced expression of *MITF* and *TYRP1* in melanocytes was observed under oxidative stress (Luo *et al.* 1994; Manga *et al.* 2006). Our previous study has shown decreased expression of *TYR, TYRP1*, and *LAMP1* in lesional and non-lesional skin (Mansuri *et al.* 2016b). In the present study, we found the down-regulation of *MITF, TYR, TYRP1*, and *LAMP1* in PIG3V melanocytes as compared to NHM and/ PIG1 melanocytes in response to CHP (Figures 4 and 5). Interestingly, *TYR* expression was significantly downregulated in PIG1 melanocytes under oxidative stress condition (Sastry *et al.* 2019). Overall, these studies advocate the reduced mRNA expression of *TYRP1* in vitiligo melanocytes with abnormal processing of TYRP1 polypeptides, which may results in increased and abnormal antigen presentation of TYRP1 peptides on melanocyte membrane leading to autoimmune response via anti-TYRP1 antibodies and/ or T cell attack on melanocytes in patients with vitiligo (Jiménez-Cervantes *et al.* 2001; Jimbow *et al.* 2001; Manga *et al.* 2006).

POLH is a member of nucleotide excision repair family genes, which encodes Pol, a specialized polymerase that is able to bypass UV lesions (Yu *et al.* 2012). When POLH is defective, UV-induced DNA

lesions are replicated by a more error-prone polymerase that produces more mutations (Flanagan *et al.* 2007). UV-induced DNA damage in melanocytes is more effectively prevented in the darker skin due to melanin (Lee *et al.* 2013). It has been reported that stimulation of melanogenesis in human melanocytes increased UVA-induced DNA damage (Denat *et al.* 2014). In conditions of vitiligo, melanocytes are under oxidative stress UV-induced DNA damage and activation of POLH is obvious. Our previous study has shown the upregulated expression of *POLH* in the lesional and non-lesional skin as well as in the blood of patients with stable vitiligo, indicating its protective role (Mansuri *et al.* 2016b). In addition, PIG3V melanocytes showed significantly increased expression of POLH in response to CHP (Figures 4 and 5).

Understanding the mechanism of H_2O_2 induced melanocyte death could elucidate the pathology underlying vitiligo in general. Melanocyte vulnerability to oxidative stress plays a vital role in the vitiligo pathogenesis. Here we have indicated that CHP promotes oxidative stress in melanocytes, and that melanocytes derived from vitiligo patients are more vulnerable to CHP induced oxidative stress as compared to normal melanocytes. We, for the first time, have demonstrated that CHP altered the expression of key genes involved in melanocyte development, oxidative stress and other cellular stress responses, making them susceptible for destruction.

5. CONCLUSION

In conclusion, vitiligo might result from an insufficient response of melanocytes to H_2O_2 induced oxidative stress. These findings will pave the way towards the development of novel therapeutic approaches for the treatment of vitiligo.

ACKNOWLEDGMENTS

We sincerely thank all the skin sample donors for participating in the study. We thank Dr. I. C. Le Poole for providing us the PIG1 and PIG3V cells. MSM and SDJ thank UGC, New Delhi for awarding SRF. MS thanks, CSIR, New Delhi for awarding SRF.

Funding: This work was supported by grants to RB from the Indian Council of Medical Research (ICMR), New Delhi, “BMS/Adhoc/122/11-2012”, Department of Biotechnology (DBT), New Delhi, “BT/PR9024/MED/12/332/2007” and Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, Gujarat, “GSBTM/MD/PROJECTS/SSA/453/2010-2011”.

Conflicts of interest: The authors declare no conflict of interest.

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How to cite this article: Mansuri MS, Singh M, Jadeja SD et.al. An in vitro study elucidating the effect of oxidative stress on melanocytes. *International Journal of Research and Review*. 2021; 8(2): 142-154.

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