Synopsis of the Ph.D. thesis on

Role of ER stress and Autoimmunity in Vitiligo Pathogenesis

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For the degree of

Ph. D. (Doctor of Philosophy) in Biochemistry

By

Shahnawaz D. Jadeja

(Registration No. FOS/1911)

Under the Supervision of

Prof. Rasheedunnisa Begum

Department of Biochemistry, Faculty of Science,

The Maharaja Sayajirao University of Baroda,

Vadodara-390002, Gujarat, India.

Introduction:

Vitiligo is one of the most common cosmetic disfigurement disorders caused due to loss of functional melanocytes from the epidermis (Le poole *et al.,* 1993). The disease can affect individuals of any race or sex and manifests before the age of 20 years in approximately half of all cases (Taieb and Picardo, 2010). Worldwide prevalence of vitiligo is about 0.06 to 2.28% of world population (Kruger *et al.,* 2012). Though, the etiology of vitiligo is complex, genetic predisposition and a number of potential precipitating factors such as oxidative stress, autoimmunity and neurological factors appear to be involved (Laddha *et al.,* 2013; Mansuri *et al.,* 2014). Various pro-oxidants generated during melanin synthesis and compromised intrinsic antioxidant defense mechanisms make epidermal melanocytes more vulnerable to oxidative stress (Denat *et al.,* 2014). Oxidative stress triggers vitiligo onset, while autoimmunity contributes to the disease progression (Laddha *et al.,* 2014). Autoimmunity plays a major role in the development of disease, as 30% of vitiligo cases are affected with at least one of the concomitant autoimmune disorders (Alkhateeb *et al*., 2003). Several studies including ours have identified critical role of $CD8^+$ cytotoxic T cells in melanocyte destruction (van den Boorn *et al*., 2009; Dwivedi *et al*., 2013). However, despite of the extensive research, the exact mechanism that connects the triggering factors with the disease progression is still obscure.

In the present study, we aim to understand the molecular events that link triggering factors with the disease progression. Oxidative stress causes disruption of cellular redox potential that extends to the endoplasmic reticulum (ER), causing accumulation of misfolded proteins, which activates the unfolded protein response (UPR). Melanocytes at the periphery of vitiligo lesions show dilation of the ER (Boissy *et al.,* 1991). Homocysteine (Hcy) is a non-protein alpha amino acid formed as an intermediate product of methionine metabolism, which is known to induce oxidative stress and is reported to be involved in various human diseases (Sharma *et al.,* 2006). Elevated levels of serum Hcy have been reported in vitiligo patients (Tsai *et al.,* 2019). Hcy causes protein misfolding in the ER resulting in UPR, leading to increased expression of immunoglobulin heavy chain-binding protein (BiP) and C/EBP-homologous protein (CHOP) (Outinen *et al*., 1999). Furthermore, UPR can contribute to the activation of autoimmune response by generation of altered self-antigens during degradation of misfolded proteins, and release of neo-antigens due to defective cell death (Todd *et al*., 2008).

The present study includes investigating genetic association of selected candidate genes such as proteasome subunit beta 8 (*PSMB8) &* transporter 1 ABC subfamily B member *(TAP1)* involved in MHC class I antigen processing and presentation; methylenetetrahydrofolate reductase *(MTHFR)* involved in Hcy metabolism; X-box binding protein-1 *(XBP1)* involved in ER stress and immune regulation; interleukin-17 *(IL17)* that mediates pro inflammatory response and tyrosinase (*TYR)* involved in melanogenesis. The study also involves estimation of plasma Hcy levels, vitamin B_{12} levels, and Hcy levels from suction induced blister fluid samples of Gujarat vitiligo patients and controls. Further, the study aims to explore the effect of Hcy on cultured Normal Human Melanocytes (NHM) in a dose and time dependent manner. The effect of Hcy on cell viability, type of cell death, ROS generation, UPR activation and induction of pro-inflammatory cytokines will be studied. Further, based on the findings of our population studies, the effect of Hcy on expression of selected candidate genes will also be monitored in Hcy treated NHM.

Objectives of the study:

1. **Investigating the association of selected candidate genes polymorphisms and their expression in Gujarat vitiligo patients and controls.**
    a) *PSMB8* intron 6 C/T (rs2071627)
    b) *TAP1* exon 10 A/G (rs1135216)
    c) *MTHFR* exon 4 C/T (rs1801133) and exon 7 A/C (rs1801131)
    d) *XBP1* -116 G/C (rs2269577)
    e) *IL17A* -197 G/A (rs2275913) and -737 C/T (rs8193036)
    f) *TYR* exon 1 A/C (rs1042602) and exon 4 G/A (rs1126809)

2. **Estimation of Hcy and vitamin B_{12} levels in Gujarat vitiligo patients and controls.**
    a) To estimate plasma Hcy and vitamin B_{12} levels in vitiligo patients and controls.
    b) To estimate Hcy levels in suction induced blister fluid samples of vitiligo patients and controls.

3. **To investigate Hcy induced ER stress in normal human melanocytes (NHM).**
    a) To monitor the dose and time dependent effect of Hcy on NHM viability
    b) To determine the type of cell death and estimate cellular ROS levels.
    c) To monitor UPR activation via PERK, IRE1 and ATF6 signaling.

d) To estimate the transcript levels of pro-inflammatory cytokines *TNFA, IFNG, IL6,* and *IL17*.
e) To estimate the transcript levels of *PSMB8, TAP1, MTHFR* and *TYR.*

Study Subjects:

Around 520 vitiligo patients and 560 age and gender matched healthy controls from S.S.G. Hospital, Vadodara, Gujarat were recruited for the population study. The diagnosis of vitiligo was based on characteristic skin depigmentation with typical localization and white color lesions on the skin, under Woods lamp. Vitiligo patients were classified into generalized vitiligo (GV) and localized vitiligo (LV) based on the distribution of patches, and active vitiligo (AV) and stable vitiligo (SV) patients based on the activity of the disease as described earlier (Ezzedine *et al.*, 2012). Neonatal foreskin samples (n=12) removed during circumcision were collected from Shifa Nursing Home, Vadodara for isolation and *in-vitro* culture of NHM. Importance of the study was explained and written consent was obtained from all the participants. The study plan and consent forms were approved by the Institutional ethical committee for human research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

<u>Objective 1.</u> Investigating the association of selected candidate genes polymorphisms and their expression in Gujarat vitiligo patients and controls.

We have studied the association of polymorphisms and expression of selected candidate genes such as *PSMB8 & TAP1* involved in MHC class I antigen processing and presentation; *MTHFR* involved in Hcy metabolism; *XBP1* involved in ER stress and immune regulation; *IL17* that mediates pro inflammatory response and *TYR* involved in melanogenesis, with vitiligo susceptibility in Gujarat population.

Analysis of PSMB8 intron 6 C/T SNP and its expression

PSMB8 intron 6 C/T *SNP* was studied in 378 vitiligo patients and 509 controls. The results suggested that the frequencies of the variant 'T' allele and homozygous 'TT' genotype of *PSMB8* intron 6 polymorphism were significantly lower in vitiligo patients as compared to controls (p=0.031 & p=0.026 respectively). Gene expression analysis revealed that *PSMB8* transcript levels (96 controls and 91 patients) and protein levels (6 controls and 6 patients)

were significantly decreased (p=0.002 and p=0.0460 respectively) in PBMCs of vitiligo patients as compared to controls.

Analysis of TAP1 exon 10 A/G SNP and its expression

Genotyping of *TAP1* exon 10 A/G SNP was carried out in 378 vitiligo patients and 509 controls. Analysis of the observed genotype and allele frequencies revealed that the occurrence of variant allele 'G' and genotype 'GG' were not significantly different in vitiligo patients and controls (p=0.470 & p=0.866 respectively). Gene expression analysis in PBMCs of 96 controls and 91 vitiligo patients revealed no significant difference (p=0.553) in *TAP1* transcript levels of vitiligo patients as compared to controls.

Analysis of MTHFR exon 4 C/T and exon 7 A/C SNPs

Both *MTHFR* SNPs were studied in 520 vitiligo patients and 558 controls. Genotyping of *MTHFR* exon 4 C/T SNP suggested that the frequencies of the variant 'T' allele and homozygous 'TT' genotype were not significantly different in vitiligo patients as compared to controls (p=0.031 & p=0.026 respectively). However, analysis of *MTHFR* exon 7 A/C SNP revealed that the frequencies of the variant 'C' allele and homozygous 'CC' genotype were significantly higher in vitiligo patients as compared to controls (p=0.037 & p=0.015 respectively).

Analysis of XBP1 -116 G/C SNP and its expression

XBP1 -116 G/C promoter polymorphism was genotyped in 146 vitiligo patients and 222 controls. The results suggested that the frequencies of the variant genotype 'GG' of *XBP1 -116* G/C SNP was significantly lower in vitiligo patients as compared to controls (p=0.036). Transcript levels of spliced and unspliced *XBP1* were estimated in PBMCs of 53 patients and 56 controls. Patients showed a significant increase in spliced *XBP1* transcript levels as compared to controls (p=0.0071). However, no significant difference in unspliced *XBP1* transcript levels was observed in patients as compared to controls (p=0.1191). The sample size for *XBP1*genetic association and expression studies will be increased further.

Analysis of IL17A -197 G/A and -737 C/T SNPs and its expression

Both the promoter polymorphisms were genotyped in 257 vitiligo patients and 292 controls. Genotyping of *IL17A* -197 G/A SNP suggested that genotype as well as allele frequencies were not significantly different (p=0.255 & p=0.238 respectively) in vitiligo patients as compared to controls. The genotype and allele frequencies of *IL17A* -737 C/T SNP were also

not significantly different in vitiligo patients and controls (p=0.057 & p=0.054 respectively). Gene expression analysis in PBMCs of 108 controls and 100 patients revealed a significant increase in *IL17A* transcript levels in vitiligo patients as compared to controls (p=0.0074).

Analysis of TYR exon 1 A/C and exon 4 G/A SNPs and its expression

TYR exon 1 and exon 4 SNPs were studied in 102 vitiligo patients and 130 controls. Genotyping of *TYR* exon 1 A/C SNP suggested that the genotype and allele frequencies were not significantly different in vitiligo patients as compared to controls (p=0.077 & p=0.515 respectively). Whereas, analysis of *TYR* exon 4 G/A SNP revealed that the frequencies of the variant 'A' allele and 'AA' genotype were significantly lower in vitiligo patients as compared to controls (p=0.011 & p=0.033 respectively). The sample size for *TYR* exon 1 and exon 4 SNPs genotyping will be increased further.

<u>Objective 2.</u> Estimation of Hcy and Vitamin B_{12} levels in Gujarat vitiligo patients and controls.

Analysis of homocysteine levels

Plasma Hcy levels were estimated in 60 controls and 55 patients. Vitiligo patients showed significantly elevated plasma Hcy levels (p=0.0003) as compared to controls. Further, analysis based on type and activity of vitiligo revealed significantly elevated plasma Hcy levels in GV as well as AV patients (p=0.0004 and p=<0.0001, respectively) as compared to controls. Analysis based on gender revealed no significant difference in plasma Hcy levels between male and female vitiligo patients (p=0.2671). Hcy levels in the skin microenvironment of vitiligo patients and controls will also be monitored by estimating Hcy levels from suction induced blister fluid samples.

Analysis of Vitamin B_{12} levels

Plasma vitamin B_{12} levels were estimated in 60 healthy controls and 55 patients. Significantly, decreased plasma vitamin B_{12} levels were observed in patients as compared to controls (p=0.0102). Moreover, analysis of different types of vitiligo suggested significantly reduced vitamin B_{12} levels in GV patients as compared to controls (p=0.033). Analysis of vitamin B_{12} levels based on the activity of vitiligo suggested a significant decrease in AV patients as compared to controls (p=0.029). No significant difference in vitamin B_{12} levels was observed between male and female vitiligo patients (p=0.3313).

Correlation of plasma Hcy levels with MTHFR exon 4 C/T and exon 7 A/C polymorphisms

MTHFR exon 4 C/T and exon 7 A/C polymorphisms were reported to show decreased MTHFR activity and influence homocysteine levels (Misra *et al*., 2010). Hence, we have analyzed the plasma Hcy levels in individuals with respect to their genotypes. Significantly elevated plasma Hcy levels were observed in vitiligo patients carrying CT+TT genotypes of *MTHFR* exon 4 C/T polymorphism and AC+CC genotypes *MTHFR* exon 7 A/C as compared to the respective ancestral genotypes (p=0.0012 and p=0.0255 respectively).

<u>Objective 3.</u> To investigate Hcy induced ER stress in normal human melanocytes (NHM).

Elevated Hcy levels in vitiligo patients led us to speculate a possible role of Hcy in melanocyte destruction. Hence, in this objective we aimed to study the various effects of Hcy on NHM.

Effect of Hcy on NHM viability

Our *in vitro* studies showed significant decrease in viability of NHM in a dose and time dependent manner upon Hcy treatment (n=3). 0.5mM Hcy showed about 15% (p=0.0034) and 25% (p=<0.0001) decrease in NHM viability at 48 and 72 hrs post treatment respectively, whereas 1mM Hcy showed about 20% (p=0.0001), 28% (p=<0.0001) and 35% (p=<0.0001) decrease in viability after 24, 48 and 72 hrs post treatment, respectively. Hcy induced cell death and generation of cellular ROS will also be monitored.

Hcy activated UPR response in NHM

Treatment of 1mM Hcy to NHM in a time dependent manner (n=3) resulted in increased splicing of *XBP1* mRNA and significantly increased expression of *BiP* and *CHOP* transcript levels (p=<0.05). We have also observed significant increase in BiP protein levels in a time dependent manner. The results suggested that Hcy causes ER stress and thereby activate UPR in NHM. We will also study the activation of UPR in NHM upon Hcy treatment by assessing the protein levels of phosphorylated eIF2α, GADD34, CHOP and ATF6 cleavage.

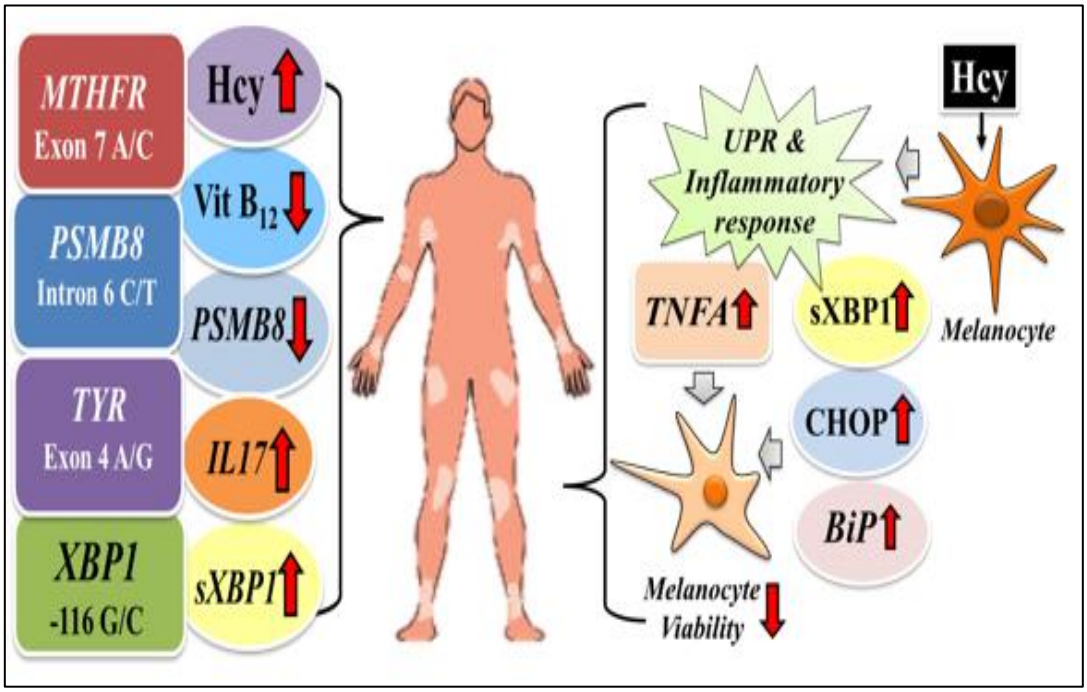
Hcy induced expression of pro-inflammatory cytokines in NHM

Interestingly, earlier reports suggested that Hcy could induce expression of various pro-inflammatory cytokines (Su *et al.*, 2005). Hence, we have monitored the effect of Hcy on expression of pro-inflammatory cytokines in NHM. Our results suggested a significant

increase in transcript levels of *TNFA* (p=<0.05) upon exposure to 1mM Hcy (n=3). Gene expression studies of *IFNG*, *IL6*, and *IL17* will also be done.

Effect of Hcy on expression of selected candidate genes in NHM

The effect of Hcy on expression of *PSMB8*, *TAP1*, *MTHFR* and *TYR* in NHM will be monitored. Gene expression analysis revealed no significant difference (p=>0.05) in *PSMB8* transcript levels in NHM treated with 1mM Hcy in a time dependent manner (n=3); transcript level analysis of *TAP1*, *MTHFR* and *TYR* will also be carried out.



Conclusion:

The present study suggests that *PSMB8*, *MTHFR*, *XBP1* and *TYR* polymorphisms; altered *IL17*, *sXBP1*, *PSMB8* transcript levels, vitamin B_{12} and Hcy levels were associated with vitiligo susceptibility in Gujarat population. Our *in-vitro* studies showed that Hcy decreased the viability, induced ER stress leading to activation of UPR as suggested by increased expression of *sXBP1*, BiP & *CHOP*. UPR also induced expression of a pro-inflammatory cytokine *TNFA* in NHM. Over all, genetic predisposition conferred by *PSMB8*, *MTHFR*, *XBP1* and *TYR* coupled with elevated Hcy could be playing a vital role in ER stress mediated melanocyte destruction in vitiligo.

Significance of the study:

The cross talk between oxidative stress, ER stress and autoimmunity appears to be crucial and may emerge as a critical aspect of vitiligo pathogenesis. The intriguing findings of the present study will help in deciphering the underlying mechanisms involved in the initial trigger by oxidative stress and contribution of ER followed by autoimmune factors in the progression of disease. The novel findings of the study in identification of genetic susceptibility loci in understanding the melanocyte-specific UPR would be of significance in developing therapies that can be used to prevent melanocyte death in vitiligo.

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Publications:

1. **Jadeja SD,** Mansuri MS, Singh M, Patel H, Marfatia YS, Begum R. (2018) Association of elevated homocysteine levels and Methylenetetrahydrofolate reductase (*MTHFR*) 1298 A > C polymorphism with Vitiligo susceptibility in Gujarat. *J Dermatol Sci* 90(2):112-122 (IF: 3.73)

2. **Jadeja SD,** Mansuri MS, Singh M, Dwivedi M, Laddha NC, Begum R. (2017) A case-control study on association of proteasome subunit beta 8 (*PSMB8*) and transporter associated with antigen processing 1 (*TAP1*) polymorphisms and their transcript levels in vitiligo from Gujarat. PLoS ONE 12(7):e0180958. **(IF:2.80)**

3. Mansuri MS, Singh M, **Jadeja SD**, Gani AR, Patel R, Dwivedi M, Laddha NC, Ansarullah, Ramachandran AV and Begum R. (2014) Could ER Stress be a Major link between Oxidative Stress and Autoimmunity in Vitiligo? J Pigmentary Disorders 1(123):2376-0427.

4. Rathwa N, Patel R, Pramanik Palit S, **Jadeja SD,** Narwaria M, Ramachandran AV, Begum R. (2019) Circulatory Omentin-1 levels but not genetic variants influence the pathophysiology of Type 2 diabetes. Cytokine. 119:144-151. **(IF: 3.51)**

5. Mansuri MS, Singh M, **Jadeja SD**, Begum R. (2018) Association of Glucose 6-Phosphate Dehydrogenase (*G6PD*) 3'UTR polymorphism with Vitiligo and in vitro studies on *G6PD* inhibition in melanocytes. *Dermatol Sci https://doi.org/10.1016/j.jdermsci.2018.12.001.* **(IF: 3.73)**

6. Singh M, Kotnis A*, **Jadeja SD***, Mondal A, Mansuri MS, Begum R. (2018) Cytokines: the yin and yang of vitiligo pathogenesis, *Expert Review of Clinical Immunol.* doi.10.1080/1744666X.2019.1550358 ***(*Both the authors contributed equally to the work)***. **(IF:3.34)**

7. Singh M, Mansuri MS, **Jadeja SD**, Marfatia YS, Begum R. (2018) Association of Interleukin 1 Receptor Antagonist (*IL1RN*) intron 2 VNTR polymorphism with vitiligo susceptibility in Gujarat population. *Ind J Dermatol Venereol Leprol* 84(3):285-291. **(IF:2.22)**

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Presentations in National/ International conferences:

1. **Jadeja SD,** Vaishnav J, Vasava J, Khan F, Narayan P, Bharti A, Begum R. "Investigating the association of Interleukin-17A (IL17A) promoter polymorphisms and its expression with vitiligo susceptibility in Gujarat population" at the Asian Advanced Course in Basic and Clinical Immunology organized by the Federation of Clinical Immunology Societies at Jaipur, Rajasthan, India on 26th – 29th March, 2019.

2. **Jadeja SD,** Vaishnav J, Narayan P, Singh M, Mansuri MS, Begum R. "Investigating the association of IL17A -197 G/A and -737 C/T promoter polymorphisms and its transcripts with vitiligo susceptibility in Gujarat population" at International Conference on 'Proteins, miRNA and Exosomes In Health and Diseases' held at The M. S. University of Baroda, Vadodara, Gujarat, India on 11th - 13th December, 2018 ***(Received the First Prize for oral presentation).***

3. **Jadeja SD,** Mansuri MS, Singh M, Patel K, Begum R. Elevated homocysteine levels and Methylenetetrahydrofolate reductase (MTHFR) 1298 A>C polymorphism are associated with vitiligo in Gujarat at 44th Annual Conference of the Indian Immunology Society (IIS) "Immunocon 2017", on 14th – 16th Dec 2017 at Institute of Science, Nirma University, S.G. Highway, Ahmedabad 382481, Gujarat ***(Awarded best poster presentation).***

4. **Jadeja SD,** Mansuri MS, Singh M, Patel H, Patel K, Begum R. "Analysis of *MTHFR* SNPs, Homocysteine and Vitamin B_{12} levels in Vitiligo cases and controls from Gujarat" at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016.

5. **Jadeja SD**, Mansuri MS, Singh M, Patel K, Begum R. "Association of *XBP*-116 C/G SNP and elevated Homocysteine levels in Gujarat Vitiligo patients- Possible implication of Homocysteine induced ER stress in Vitiligo" at International Conference on Genomic Medicine in Skin Research on 24th and 25th June 2016 held at CSIR-IGIB, New Delhi, India.

6. **Jadeja SD**, Mansuri MS, Singh M, Ansarullah, Patel H, Begum R. "Investigating association of two genetic variants of *MTHFR* (677 C>T and 1298 A>C) with Vitiligo Susceptibility in Gujarat Population" at the Master Class on Vitiligo and Pigmentary Disorders and 2nd Annual meeting of Vitiligo Academy of India, 28th – 30th November, 2014 held at Amritsar, India.

7. **Jadeja SD**, Mansuri MS, Singh M, Laddha NC, Dwivedi M, Begum R. "Association of *LMP7* and *TAP1* polymorphisms with Vitiligo susceptibility in Gujarat population" at the International Conference on XXII International Pigment Cell Conference, 4th -7th September, 2014 held at Singapore ***(Received the ICMR International Travel Award for Poster Presentation).***

Participation in Workshops/ Seminars:

1. Participated in the ***"Asian Advanced Course in Basic and Clinical Immunology"*** organized by the Federation of Clinical Immunology Societies at Jaipur, Rajasthan, India on 26th – 29th March, 2019.

2. Participated in workshop on ***"Melanocyte: from Bench to Bedside"*** at CSIR-IGIB, New Delhi, India on 23rd June 2016.

3. Participated in Wellcome Trust/ DBT India sponsored workshop on ***"Science Communication"*** at the Department of Microbiology and Biotechnology Centre, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India on 11th March, 2016.

4. Participated in workshop on ***"In vitro: Art and Science of Cell Culture"*** at Institute of Science Nirma University, Ahmedabad from 2nd to 4th February, 2016.

Date:

Jadeja Shahnawaz D.
Ph. D. candidate

Prof. Rasheedunnisa Begum
Guide

Head
Department of Biochemistry

Dean
Faculty of Science