REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1.	Name and designation of ICMR- IF	Dr. Dipty Singh
2.	Address	Neuroendocrinology, ICMR-NIRRH, Parel, Mumbai-12
3.	Frontline area of research in which training/research was carried out	Male Infertility
4.	Name & address of Professor and host institute	Dr. Ashok Agarwal American Center for Reproductive Medicine Cleveland Clinic | Mail Code X-11 | 10681 Carnegie Avenue |Cleveland | OH 44195 | United States |
5.	Duration of fellowship	6 Months (8th January 2019 to 5th July 2019)
6.	Highlights of work conducted **i.Technique/expertise acquired** **Training Obtained:** Undergone andrology training for semen analysis and advance sperm function tests as per 2010 World Health Organization Criteria at Andrology Center, Cleveland Clinic **Protocol/ Assay Developed:** 1. Evaluation of sperm mitochondrial membrane potential (MMP) by flow cytometry 2.Evaluation of sperm intracellular reactive oxygen species (iROS) by flow cytometry **Techniques Learnt:** 1. Flow cytometry; 2. Sperm selection techniques; 3. Sperm cryopreservation; 4. ORP test – MiOXSYS™ system; 5. TUNEL assay;	

ii. Research results, including any papers, prepared/submitted for publication

Summary of work done:

Title: Association of Sperm Mitochondrial Dysfunction and mtDNA Methylation Changes in Infertile Men with Clinical Varicocele - A Pilot Study

Varicocele is a leading cause of male infertility. Current evidence indicates that mitochondrial dysfunction is a key contributing factor to the mechanism by which varicocele impairs sperm function. Mitochondrial membrane potential (MMP) and intracellular reactive oxygen species (iROS) are prime indicators of mitochondrial dysfunction. Recent studies also indicate the perturbed gene expression in spermatozoa of men with varicocele. Epigenetics plays an important role in the regulation of gene expression through modulation of DNA activity without altering the basic nucleotide structure. Very few studies have reported epigenetic changes in infertile men with clinical varicocele. Therefore, the objective of this study was to evaluate mitochondrial dysfunction and mtDNA methylation changes in spermatozoa of men with clinical varicocele.

This case-controlled study included 9 infertile men with unilateral and bilateral varicocele (grade II–III), and 9 healthy fertile men of reproductive age. Routine semen parameters (2010 World Health Organization Criteria) were analyzed following liquefaction. MMP (JC-1 dye) and iROS (2',7'-dichlorofluorescin diacetate, DCFDA) assays were performed using an Accuri C6 flow cytometer (Becton and Dickinson, San Jose, CA) after sperm separation with a 65% density gradient. Oxidation-reduction potential (ORP) was measured by MiOXSYS (Male Infertility Oxidative System). Furthermore, the sperm DNA from healthy fertile men and infertile varicocele men were isolated using QIAamp DNA mini kit (Qiagen, Hinden,Germany) for methylation studies. Sperm DNA fragmentation was evaluated using a terminal deoxynucleotidyl transferase–mediated fluorescein-TUNEL assay with an Apo-Direct kit (Pharmingen, San Diego, CA). Statistical analysis was carried out with an independent samples t-test using MedCalc Statistical Software (version 17.8; Ostend, Belgium).

Our results indicated that infertile men with varicocele had significantly lower sperm count and motility compared to healthy fertile donors. Patients with varicocele had a significantly lower MMP than healthy fertile donors. iROS produced by live spermatozoa

and seminal ORP levels were significantly higher in men with varicocele. There is a comparable positive correlation (r=0.238; P=0.5702) between ORP and iROS. Sperm viability was significantly lower in varicocele patients. Decreased MMP and increased iROS indicate sperm mitochondrial dysfunction in men with varicocele. The correlation between oxidative stress and mitochondrial dysfunction in spermatozoa supports its crucial role in the pathogenesis of varicocele-associated male infertility. This study further supports the role of mitochondrial dysfunction as a central mechanism of varicocele-related male infertility.

Title: Impact of deep-freezing and cryopreservation on mitochondrial membrane potential and intracellular reactive oxygen species production in human spermatozoa

Human spermatozoa are very susceptible to cryodamage, which is partly caused by reactive oxygen species (ROS) and results in poor sperm quality and altered functional capacity after thawing. The main objective of this study was to evaluate the effect of deep freezing (-80°C) and cryopreservation (-196°C) on mitochondrial membrane potential (MMP) and intracellular reactive oxygen species (iROS) generation in human spermatozoa.

Healthy fertile donors (n=7) of reproductive age with normal semen parameters according to 2010 World Health Organization Criteria were included in the study. Routine semen analysis was performed on fresh samples after liquefaction. Following sperm separation using a 65% density gradient, one aliquot of the sample was deep-frozen at -80°C and another aliquot was cryopreserved at -196°C. Samples were thawed at 37°C for 20 min. MMP (JC-1 dye) and iROS (2',7'–dichlorofluorescin diacetate, DCFDA) assays were performed on fresh, deep-frozen and cryopreserved samples using an Accuri C6 flow cytometer (Becton and Dickinson, San Jose, CA). Statistical analysis was carried out with a paired t-test using MedCalc Statistical Software (version 17.8; Ostend, Belgium).

Our results revealed that the MMP levels significantly reduced in deep-frozen and cryopreserved samples versus the fresh samples. However, cryopreserved samples showed higher intact MMP spermatozoa compared with deep-frozen samples. Fresh samples had significantly lower levels of iROS as compared with deep-frozen and cryopreserved samples. There was a significant overall inverse correlation observed between iROS and MMP levels. The overall MMP had significant positive correlation with sperm motility. Deep-freezing and cryopreservation negatively impact the MMP and iROS levels which

	leads to compromised quality of spermatozoa. Our results indicated that MMP and iROS levels effectively reflect sperm quality. Since, cryopreservation of sperm is a widely used technique for infertility treatment; future research may focus on reducing cryodamage and thereby improving sperm quality. **Publication as First Author:** D. Singh, S. Gupta, M.K. Panner Selvam, R. Henkel, R. Sharma, A. Agarwal. Impact of deep-freezing and cryopreservation on mitochondrial membrane potential and intracellular reactive oxygen species production in human spermatozoa. **(Manuscript to be communicated)** **Publication as Co-Author:** Saradha Baskaran, Ashok Agarwa, Manesh Kumar Panner Selvam, Ralf Henkel, Damayanthi Durairajanayagam, Kristian Leisegang, Ahmad Majzoub, Dipty Singh, Kareim Khalafalla. Is There Plagiarism in the Most Influential Publications of Andrology? **(Under revision in Andrologia journal)**
	iii.Proposed utilization of the experience in India: The new techniques learnt will be very useful for the successful and effective implementation of the research projects being carried out at the institute in the area of male infertility. The overall research experience gained and experimental skills acquired at American Center for Reproductive Medicine, Cleveland Clinic, USA will be helpful in developing new research program in the area of Varicocele induced male infertility at ICMR-NIRRH.

23/7/2019

Signature of ICMR-IF

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