IMPACT OF MITOCHONDRIAL TRANSPLANTATION ON CANCER CELLS

A Dissertation submitted to the Central University of Punjab

For the Award of

Master of Science

In

Biosciences

By

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DECLARATION

I declare that the dissertation entitled "**IMPACT OF MITOCHONDRIAL TRANSPLANTATION ON CANCER CELLS**" has been prepared by me under the guidance of Dr. Pankaj Bhardwaj, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab and Dr. Sandeep Singh, Assistant Professor, Centre for Genetic Diseases and Molecular Medicine, School of Emerging Life Science Technologies, Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

It is to certify that **ALZA AGGARWAL** has prepared her dissertation entitled "**IMPACT OF MITOCHONDRIAL TRANSPLANTATION ON CANCER CELLS**", for the award of M.Sc. degree of the Central University of Punjab. She has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

IMPACT OF MITOCHONDRIAL TRANSPLANTATION ON CANCER CELLS

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Keywords : Mitochondria, Mitochondrial dysfunction, Cancer, Carcinogenesis and Drug Sensitivity.

Mitochondria, the powerhouse of the cell, are small granular or filamentous bodies associated significantly with cellular respiration and are the main sources of energy, due to which they are present in maximum number in the organs that require large amounts of energy for doing their function like muscle cells, neural cells, etc. In case of any dysfunction of mitochondria, these organs are most affected culminating in a number of serious multi organs diseases, irrespective of age such as neurogenic weakness with ataxia and retinitis pigmentosa (NARP), or Leigh syndrome (LS), Cancer, etc. Although mutations in mitochondrial genes are common in cancer cells, they do not inactivate mitochondrial energy metabolism, but rather alter the mitochondrial bioenergetics and biosynthetic state. Literature survey also revealed that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients. This study is focused on the impact of mitochondrial transplantation on cancer cells and their drug sensitivity against four human cancer cell lines HCT116 (WT & P53mutated), HepG2 and MCF7. The normal cell's Mitochondria was transplanted into cancer cells and then evaluated the Impact of transplantation of mitochondria from healthy cells into cancer cell upon their growth, ROS production and their drug sensitivity. The results of this study revealed that the healthy mitochondria transplanted to cancer cells decrease carcinogenesis and have drug sensitivity. So, it may be used as futuristic cancer remedy.

(Signature of Student) (Signature of Supervisor)

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Dimethyl sulfoxide	DMSO
2	Fetal Bovine Serum	FBS
3	Duldecco's Modified Eagle's Serum	DMEM
4	Phosphate Buffer Solution	PBS
5	Ethylene diaminetetra acetic acid	EDTA
6	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	HEPES
7	(3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide)	MTT
8	(5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi-dazolylcarbocyanine iodide)	JC-1
9	Dihydro dichlorofluroscien diacetate	H_2DCFDA
10	Thiobarbituric Acid	TBA
11	Tri Chloroacetic Acid	TCA
12	Malondialdehyde Tetraethyl Alcohol	MDA
13	Hydrogen peroxide	H_2O_2
14	Hydrochloric acid	HCl
15	Magnesium chloride	$MgCl_2$
16	Sodium hydroxide	NaOH
17	Potassium dihydrogen phosphate	KH_2PO_4

18	Potassium chloride	KCl
19	Disodium hydrogen phosphate	Na_2HPO_4
20	Nicotinamide adenine dinucleotide phosphate	NADPH
21	Superoxide dismutase	SOD
22	Microliter	µl
23	Milliliter	ml
24	Molar Concentration	M

CHAPTER- 1

INTRODUCTION

The human body, organized at several levels, from the simplest to the most complex, encompasses within itself numerous cells, organs and tissues each of which is having numerous crosstalk amongst all the hierarchical level to maintain homeostasis. Cells are the fundamental unit of life, made up of molecules and organelles [Cooper et al., 2000]. Each type of cell is specially adapted to perform its specific function. The human body at large consists of 11 organ system (digestive system, respiratory system, reproductive system, etc.) which together assist in maintaining homeostasis of the human body [Silverthorn et al., 2009]. These organ systems are in turn made up of groups of organs, which are formed as a consequence of tissues of similar function combining together. Tissues, on other hand, result from aggregation of similar cells. Cells, known as chemical factories, are surrounded by a plasma membrane and within this membrane lies the cytoplasm composed of fluid and organelles of the cells, such as endoplasmic reticulum, ribosomes, golgi apparatus, vacuoles, mitochondria etc. These organelles are critical for cellular function. For instance mitochondria is the one of most important organelle of the cell [Lill et al., 2000], without which more than 95% of the cell's energy, which releases nutrients, would cease immediately [Guyton et al., 2007].

Mitochondria, the powerhouse of the cell, are small granular or filamentous bodies [McBride et al., 2006] associated significantly with cellular respiration and are the main sources of energy. These structures known as the engine of the cell was first observed, as granular structures in striated muscle, by the German biologist Rudolph Kolliker in 1850 [Powar, 2010; Albert et al., 2010]. But, it was not until 1898, that scientist Bends developed the crystal violet staining technique and called the structures mitochondria. Dimensionally, the average length of the mitochondrion is 3-4 microns and the average diameter being 0.5 to 1.0 microns. In plants they alter their shape and volume [Powar, 2010; Albert et al., 2010].The main function of mitochondria is the generation of ATP, due to which they are present in maximum

number in the organs that require large amounts of energy for doing their function like muscle cells, neural cells, etc. In case of any dysfunction of mitochondria, these organs are most affected culminating in a number of serious multiorgan diseases, irrespective of age [Kispa et al., 1999; Pagon et al., 2000; Wallace, 2001; Wallace, 2012].Although, mitochondrial disorders may affect only a single organ (e.g., the eye in Leber hereditary optic neuropathy [LHON]), they may involve multiple organ systems often present with prominent neurologic and myopathic features such as e.g., Kearns-Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), neurogenic weakness with ataxia and retinitis pigmentosa (NARP), or Leigh syndrome (LS), Cancer [Pagon et al. 2000]. Functional mitochondria are observed to be pivotal for the survival of the cancer cell and a decrease in mitochondrial energy metabolism might lead to the development of cancer [Wallace, 2012; Gogvadze et al 2008].Cancer cells generally demand higher energy and, thus, targeting the cellular energy source [Weinhouse, 1976] may prove to be a potent anticancer therapy. Although mutations in mitochondrial genes are common in cancer cells, they do not inactivate mitochondrial energy metabolism, but rather alter the mitochondrial bioenergetics and biosynthetic state [Wallace 2012]. Some studies show that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients [Lai et al., 1991; Joly et al., 2002]. This may result because of resistance to mitochondrial membrane permeabilization (MMP). [Rustin et al., 2008]. Subsequently, the release of pro-apoptotic proteins from the mitochondrial intermembrane space is inhibited. In addition, resistance may also arise from the down regulation of pro-apoptotic factors (proteins or second messengers such as calcium and reactive oxygen species (ROS) and up-regulation of anti-apoptotic proteins, thus, tilting the balance toward survival [Indran et al., 2011].

CHAPTER- 2
REVIEW OF LITERATURE

2.1 Mitochondria and its origin- Mitochondrial origin is said to be based on the endosymbiotic theory postulated by Lynn Margulis in the 1967 [Lodish et al., 2003; Witzany, 2006]. This theory proposed that-

- Aerobic bacteria engulf anaerobic bacteria.
- Mitochondria are the result of endocytosis of aerobic bacteria.
- Chloroplasts are the result of endocytosis of photosynthetic bacteria.
- This arrangement became a mutually beneficial relationship for both cells (symbiotic), because the aerobic bacteria would have handled the toxic oxygen for the anaerobic bacteria, and the anaerobic bacteria would ingested food and protected the aerobic "symbiotic".
- Thus, a cell with a double-membrane bound organelle, mitochondria resulted. The inner lipid bilayer of mitochondria came from the bacterial cell's plasma membrane, and the outer lipid bilayer came from the cell that engulfed it [Nardon et al. 1991; Lodish et al., 2003; Witzany, 2006].

2.2 Biogenesis of mitochondria- Biogenesis involves the division of the mitochondria into two parts which then subsequently grows independently. It involves multiple steps wherein the peroxisome proliferators-activated receptor gamma co-activator (PGC-1α) activates nuclear transcription factors (NTFs) leading to transcription of nuclear- encoded proteins and of the mitochondrial transcription factor, Tfam, which is then transported to mitochondria [Karp 2010]. In mitochondria, Tfam activates transcription and replication of the mitochondrial genome. Nuclear-encoded proteins are imported into mitochondria through the outer (TOM) or inner (TIM) membrane transport machinery [Karp 2010]. Nuclear- and mitochondria-encoded subunits of the respiratory chain are then assembled. Mitochondrial fission through the dynamic-related protein 1 (DRP1) for the outer membrane and OPA1 for the inner membrane of mitochondria allow mitochondrial division, while mitofusins (Mfn) control mitochondrial fusion [Tedeschi et al., 1976; Attardi et al., 1988; Kispal et al., 1999]

2.3 Structure of mitochondria- The mitochondria are bound by two membranes (Figure 2.1), the outer and inner membrane [Frezza et al., 2009], separated by space known as the inter membrane space. This space, being 40-70 nm in width, is filled with a fluid. The outer membrane is permeable in nature and the space bound by the inner chamber is called inner chamber or inner membrane space. The inner membrane is filled with a matrix which contains dense granules, ribosome and mitochondrial DNA [Albert et al., 2010; Sherratt 1990; Frey et al., 2000].

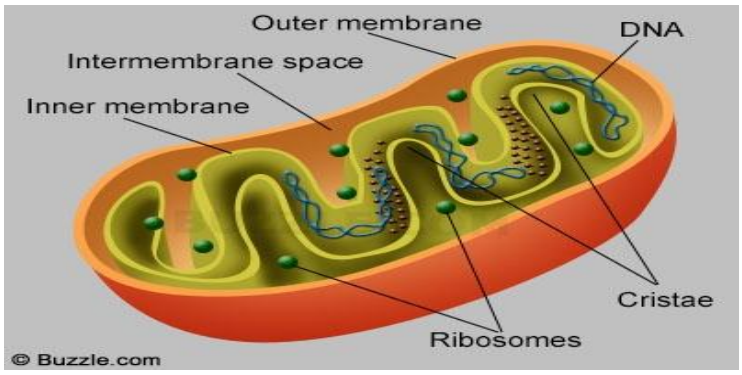


Fig- 2.1 Structure of mitochondria

(Source: http://www.buzzle.com/articles/mitochondria-structure-and-functions.html)

2.4 Functions of mitochondria- Mitochondria are the most important organelle in eukaryotic cells, carrying out a wide variety of biochemical processes, depending on the cell in which they are present. In general, mitochondrial function varies from tissue to tissue. These encompass cardinal functions like breaking down, building up and recycling the products needed to do other functions. They are required for cholesterol metabolism, neurotransmitter metabolism, and also help in the detoxification of ammonia in the urea cycle. Dysfunctioning of Mitochondria not only affects the energy metabolism, but also affects the cell specific functioning of normal cell [Hardin et al., 2012]. In addition to these, there are three more important aspects of mitochondria: (i) energy production, (ii) generation of reactive oxygen species (ROS) and (iii) regulation of programmed cell death, or apoptosis [Tedeschi et al., 1976; Wallace, 1999].

a) **Energy production-** The most important function of mitochondria is energy production in the form of ATP. The raw materials are food materials and tissues which are broken down in catabolism which are then transferred to mitochondria for further processing. In inner membrane they have electrical charges which help in the production of ATP (Phosphorylaton), by combining with oxygen (oxidation) through the five electron transport chain complexes, in a process called oxidative phosphorylation [Albert et al., 2010]. According to Fu et al. (2012), the translocation of Cystathionine γ-lyase (CSE) to mitochondria on specific stress stimulations is a unique mechanism to promote H_2S production in mitochondria, which subsequently sustains mitochondrial ATP production under hypoxia conditions [Fu et al., 2012; Wallace, 1999].

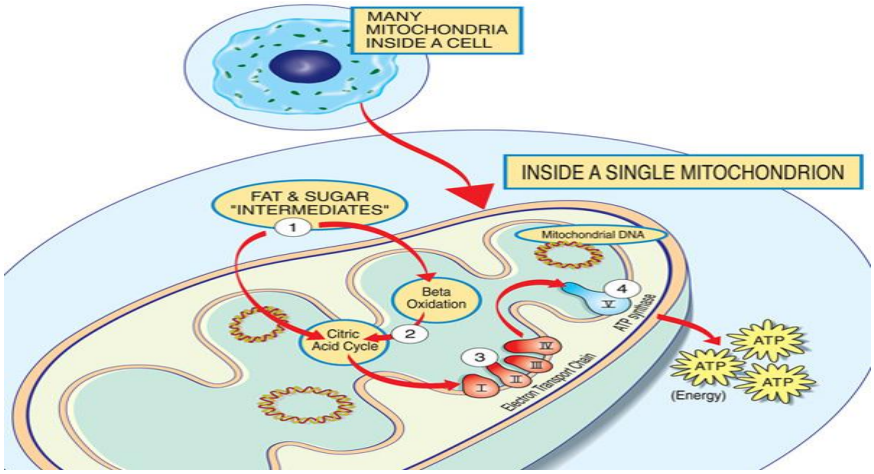


Fig-2.2 Energy productions in mitochondria

(Source: http://www.mitoresearch.org)

b) **Generation of reactive oxygen species (ROS) -** A large portion of ROS is produced in the mitochondria through metabolic processes. Superoxides are the primary radical produced. It is dismutated to hydrogen peroxide through superoxide dismutase. At normal cellular levels, ROS play an important role in cell signaling in the cell. However, under hypoxic conditions, ROS induce excess damage to cellular components, including proteins, lipids, and DNA [Wallace, 1999; Wallace et al., 2010].

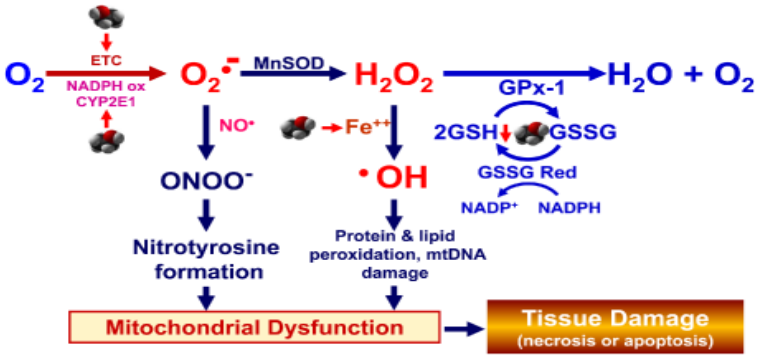


Fig- 2.3 Generation of reactive oxygen species

(Source: http://hepatitiscnewdrugs.blogspot.in)

c) **Programmed cell death-** This process helps in generating immune response to illness, an important function of the mitochondria [Alberts et al., 2010], ultimately resulting in destruction of the unwanted products formed during the development of organism. During abnormal conditions, when mitochondria do not carry out their function, more oxygen is used up to make ATP, in the process producing free radicals which damage the proteins, DNA as well as the membranes of mitochondria. The mitochondrial inner membrane space contains a number of cell death-promoting factors, including cytochrome c, apoptosis-inducing factor (AIF, a flavoprotein), and latent forms of specialized proteases called procaspases. Opening of the mitochondrial permeability transition pore (mtPTP) causes swelling of the mitochondrial inner membrane, and release of these deaths-promoting factors which leading to the destruction of the cytoplasm [Wallace, 1999].

2.5 Mitochondrial genome

Mitochondria are unique organelles in animal cells as they contain their own DNA. It was sequenced in 1981 by Anderson. The number of mitochondria and mitochondrial DNA (mtDNA) varies from tissue to tissue. The human mitochondrial genome is a small circular DNA molecules which have 16.569 kb circular DNA that containing 37genes which are 22 tRNAs, 2 rRNAs, 13 polypeptides [Bernt et al., 2013; Shadel et al., 1997]. Mitochondria genome is situated within the mitochondrial matrix and present in thousands of copies per cell. Although, mtDNA represents less than 1% of

total cellular DNA, its gene products are essential for normal cellular function. Mitochondrial DNA has two strands, a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand. The heavy strand contains 12 of the 13 polypeptide-encoding genes, 14 of the 22 tRNA-encoding genes and both rRNA-encoding genes [Anderson et al., 1981; Taanman et al., 1999]. In human mitochondrial genome, Introns are absent. There are two types of sequences in mitochondrial genome coding and non-coding sequences.The only non-coding segment of mtDNA is the displacement loop that is called D-loop, a region of 1121 bp that contains the origin of replication of the H-strand (O_H) and the promoters for L and H-strand transcription. The mtDNA is replicated from two origins O_H and O_L. The human mitochondrial genome encodes 13 subunits of respiratory chain complexes: seven subunits (ND 1–6 and 4L) of complex I, cytochrome b (Cyt b) of complex III, the COX I–III subunits of cytochrome oxidase or complex IV, and the ATPase 6 and 8 subunits of F_OF_1 ATP synthase. MtDNA also encode 12S and 16S rRNA genes and 22 tRNA genes [Anderson et al., 1981]. The abbreviated amino acid names specify the subsequent amino acid tRNA genes. The outer strand is heavy-chain DNA and the inner one light-chain DNA, While P_H and P_L indicate the transcription sites. In fact, the mutation frequency in mtDNA is approximately tenfold greater than that in nuclear DNA. MtDNA within a single cell generally has identical sequences, which is described as homoplasmic. Heteroplasmy can occur in response to somatic mutations. Since, there can be thousands of copies of mtDNA within a single cell.

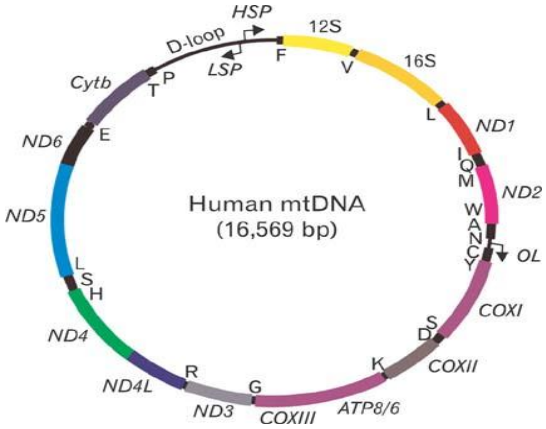


Fig- 2.4 Mitochondrial genome

(Source:http://acbjournal.org/search.php)

Mitochondria are the powerhouses of cells, but also contain the mechanisms leading to cell death. It plays an important role in multiple cellular processes including energy metabolism, cell death and aging. It regulates energy production maintaining energy homeostasis in normal cells and functional organs. However, mitochondria go through a series of morphological and functional changing during carcinogenesis. The mitochondrial cell transformation alters their fast proliferation and resistance to cell death and facilitates metastasis. These transformations provide targets for anticancer treatment at different levels [Wang et al., 2013]. Functional mitochondria are essential to the cancer cell. Although, mutations in mitochondrial genes are common in cancer cells, they do not inactivate mitochondrial energy metabolism, but rather alter the mitochondrial bioenergetics and biosynthetic state [Wallace et al., 2012]. Mitochondrial cells activate the cell death machinery in cancer cells by stimulating mitochondrial membrane permeabilization which is promising therapeutic approaches for cancer treatment [Fulda et al., 2010]. Cancer-associated changes in cellular metabolism (the Warburg effect) influence mitochondrial function, and the invalidation of apoptosis is linked to an inhibition of mitochondrial outer membrane permeabilization (MOMP); [Galluzzi et al., 2006]. Mitochondrial structure and function might prove clinically useful either as markers for the early detection of cancer or as unique molecular sites against selective chemotherapeutic agents [Josephine et al., 2002].

In cancer cells, the structure and function of mitochondria is different from normal cells [Josephine et al., 2004]. Cancer cells have decreased mitochondrial activity and shift to aerobic glycolysis for ATP production, a phenomenon known as the Warburg effect [Gogvadze et al., 2008]. Cancer cells are often more resistant to activation of the mitochondrial apoptotic pathway due to over expression of antiapoptotic Bcl-2 family proteins [Chen et al., 2010] or stabilization of the mitochondrial membrane against apoptosis-associated permeabilization [Kroemer et al., 2008]. Cancer cells have increased ROS level due to mitochondrial dysfunction [Brandon et al. 2006]. Therefore, it is possibility that cancer cells have a lower tolerance capacity when they are further oxidized by ROS generating drugs. A direct induction of apoptosis in cancer cells via the mitochondrial pathway allows one to more upstream signal

transduction steps frequently impaired in human cancer [Guizzunti et al., 2012].This mitochondria may help to improve the poor results of traditional therapies and represent a promising approach for the treatment of cancer cells resistant to standard chemotherapy [Fulda et al., 2010].

A number of metabolic aberrations in cancer cells have been found to be specifically associated with mitochondrial metabolic function which include altered enzyme activities or functions of proteins in the mitochondria, as well as membrane potential, composition and expression of mitochondrial proteins. Due to decreases in the number of mitochondria and changes of mitochondrial morphology, age-related neurodegenerative diseases are occurring. Electron transport chain decreased in cancer cells. Some of these changes, such as increased cholesterol content, may be associated with increases in hexokinase, which shift toward glycolytic metabolism. Stoichiometries mismatches in OXPOHOS complexes lead to altered OXPHOS function [Gogvadze et al., 2008]. These alterations occur as a result of the accumulation of mutations in the mtDNA [Wallace et al., 2010]. This accumulation of mtDNA mutations are known to play a significant role in the pathogenesis of many age-related neurodegenerative diseases and age-related pathological alterations of heart, skeletal muscle and the vascular system. Alterations of mtDNA have been also correlated with tumor progression and have been reported in a variety of cancers, including ovarian, thyroid, salivary, kidney, liver, lung, colon, gastric, brain, bladder, head and neck, and breast cancers [Desler et al., 2011]. Alternations may occur as a consequence of point mutations, deletions, depletion or merely tumor progression [He et al., 2010]. According to Frezza (2014), mitochondria play a pivotal role in shaping the oncogenic signalling cascade and that mitochondrial dysfunction, in some circumstances, a primary step for cancer transformation.

The functional significance of mtDNA mutation and depletion may be a useful marker of tumor progression. The abundance and homoplasmic nature of mitochondria make mtDNA an attractive molecular marker of cancer [Gogvadze et al., 2008]. Mutations in mtDNA content may lead to cancer [Gogvadze et al., 2008] with the D-loop region shown to be a mutational hotspot in human cancer. This may be owing to the fact that

D-loop region regulates elements involved in mtDNA replication and the resulting mutation in the D-loop may affect the mtDNA copy number which is observed to increase or decrease during cancer development [Gogvadze et al., 2008]. Signaling pathways, that lead to apoptosis also affected in the gene regulation process. The expression of BAX, a proapoptotic inner mitochondrial protein, is observed to reduce in some cancer cells. Another pathway involved in cancer progression, which is affected by mitochondrial metabolism is the stabilization of HIF-1α, which degrade in the presence of oxygen. HIF-1 suppresses mitochondrial function in tumor cells, suggesting that it modulates the reciprocal relationship between glycolysis and oxidative phosphorylation [Gogvadze et al., 2008]. Mitochondrial replication requires the capacity to replicate and express mtDNA, and this mitochondrial function depends predominantly on the impermeability of the inner membrane and the catalytic integrity of the respiratory complexes. The gradually reducing mitochondrial function in various tissues, results in muscle and liver toxicity, as well as lipodystrophy and lipoatrophy [Lai et al., 1991; Joly et al., 2002]. Some drugs, such as certain aminoglycoside antibiotics, induce long-term mitochondrial dysfunction by impairing protein synthesis in the organelle, resulting in ototoxicity and nephrotoxicity [Fischel and Ghodsian, 2005]. Other drugs, such as acetaminophen, doxorubicin, and ethanol impose oxidative stress via redox cycling or via glutathione depletion, cyp2E1-derived ROS generation, or reactive metabolite formation, such free radicals can directly inactivate several of the electron transport complexes, but more circuitous routes to mitochondrial failure are also important [Wallace, 2003; Dykens et al., 2007].

2.5 Statement of the Problem

Since, it is already known that mitochondria play a central role in carcinogenesis via not only through altered metabolism but also through regulation of apoptosis. Many failed clinical trials for anticancer drugs are also attributed to quality of mitochondria in those cancers. In nutshell it may be concluded that altered mitochondria functioning not only attributes to tumorigenesis but also render anticancer drugs ineffective. Such alterations in mitochondria may be reversed by transplantation of mitochondria from normal cells. Such organelle transplantation may result in reversal of cancer related

metabolic changes as well as increases chemo-sensitivity of cancer cells. In light of our current understanding the study proposes following objectives:

2.6 Objectives

The information obtained from the literature survey encouraged us to propose the research project with the following aims:

I. Mitochondrial transplantation into cancer cells using various methods and to assess their efficiency.

II. Impact of transplantation of mitochondria from healthy cells into cancer cell upon their growth, ROS production and other biochemical parameters.

III. Evaluation of sensitivity of transplanted cancer cells towards various anticancer drugs compared to the cancer cells.

CHAPTER- 3
MATERIALS AND METHODS

3.1 Material

3.1.1 Cell culture media, trypsin, heat inactivated FBS (Fetal Bovine Serum), antibiotic solutions Ciprofloxacin Penstrip, MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide), JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimi- dazolylcarbocyanine iodide), PBS (Phosphate Buffer Solution), DMSO (Dimethyl sulfoxide**)**, H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate), Agar, HCl (Hydrochloric acid), Homogenizing buffer [KCl (Potassium chloride), $MgCl_2$ (Magnesium chloride),HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EDTA (EthyleneDiamineTetra Acetic acid)], NaCl (Sodium chloride), Na_2HPO_4 (Disodium hydrogen phosphate), KH_2PO_4 (Potassium dihydrogen phosphate), etc. were purchased from Invitrogen and Loba.

3.1.2 Instruments

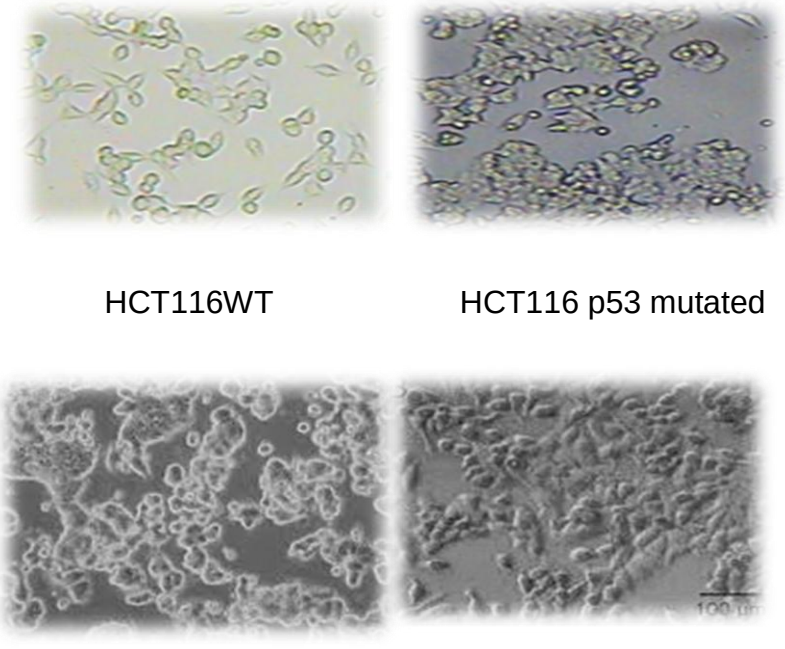
Table-1: Various instruments used in the entire Research work have been listed below in the table-

Sr. No.	Name of Instrument	Manufacturing Company
1.	Analytical Balance TE214,S	Sartorius
2.	Autoclave (vertical) NSW-227	Calton Company
3.	Inverted Microscope with live Imaging Facility	Olympus Magnus
4.	Light Binocular Microscope	Olympus Magnus
5.	Horizontal Laminar air flow NSW-201	Calton
6.	CO_2 Incubator	New Brunswick, UK
7.	Mini Centrifuge MC-02	SpinwinDaikan Scientific Co.Ltd.
8.	pH Meter	Mettler Toledo
9.	Rectangular water bath	New India
10.	Refrigerated Centrifuge 5430R	Eppendorf, Germany
11.	Microplate Reader Synergy H1	Biotek
12.	Ultrasonic Cleaner	Citizon

13.	Hot plate	Tarsons
14.	UV-VIS double beam 2202 Spectrophotometer	Systronics
15.	Electrophoresis Unit	Tarsons
16.	Dancing Shaker	Tarsons

3.1.3 Cell lines

Cancer cell line used in the research work to study the biology of cancer was mainly HCT 116 Colon Cancer Cell line (HCT-116 wild type, HCT-116 p53 Null type), MCF7 Breast Cancer Cell line, and HepG2 liver cancer cell line, as kind gift, obtained from Prof. Tapas MukhUpadhyay, Former Director, National Center for Human Genome Studies and Research (NCHGSR), Punjab University, Chandigarh. During this research work I would try to see the Impact of transplantation of mitochondria from healthy cells into cancer cell upon their growth, metastatic potential, ROS production and other biochemical parameters.



HCT116WT HCT116 p53 mutated

MCF7 HepG2

Fig- 3.1 Microscopic appearance (at20X) HCT116 WT, HCT116p53 mutated, MCF7 and HepG2 Cell lines.

3.1.4 Methods

The following plan was charted out and accordingly carried out:

Experiment 1: Mitochondrial Isolation and Transplantation

Experiment 2: Evaluation of cell viability in cell Suspension

-MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] Assay

-Dye Exclusion Assay

Experiment 3: Evaluation of colony forming ability of cells by CFU (Colony Forming Unit) Assay

Experiment 4: Evaluation of Mitochondrial Membrane Potential

Experiment 5: Assessment of the oxidative stress in the cells

-H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate)

-Lipid peroxidation (EC 1.11.1.11)

Experiment 6: Assessment of Antioxidant Enzymes Activity in Cell

-SOD (Sodium dismutase) Assay (EC 1.15.1.1)

-Catalase Activity (EC 1.11.1.6)

-Glutathione Reductase (EC 1.6.4.2)

Experiment 7: Evaluation of Drug Sensitivity in cells

3.2. Routine Assay in cell culture lab

Culturing of the cell lines

The monolayer of cells was detached by trypsinization carried out for 4 min. This trypsin was inactivated by adding 1ml of media containing serum. Subsequently, the cells were harvested by centrifugation at 1200 rpm for 10 minutes. The supernatant was discarded and resuspended the cell pellet in 2ml of complete media. Then, the cells were cultured in fresh media. Then cells were observed under inverted microscope and incubated at 37^0C in CO_2 incubator

Maintenance and Sub Culturing of cell lines

Cell lines were cultured and maintained in 25cm^2 flasks containingDMEM media supplemented with 10% FBS, 1X Penstrip, and incubated at 37^oC in a humidified atmosphere containing 5% CO_2. Sub culturing was done in the 25cm^2 culturing flask.

The monolayer of cells was detached by trypsinization for 4 minutes. Then, the trypsin was inactivated by adding 1ml of media containing serum. Then, the cells were harvested by centrifugation at 1200 rpm for 10 minutes. The supernatant was discarded and resuspended the cell pellet in 2ml of complete media and transferred to $25cm^2$ or $75cm^2$ flasks which containing 5ml of the complete media. The cells were cultured by giving fresh media for every three days. Then, the cells were observed under inverted microscope and incubated at 37^0C in CO_2 incubator.

Cryopreservation and thawing of the cell lines

Cell lines were preserved by freezing cryovials. Cell lines were rapidly thawed at $37^0C$and then resuspended in 4ml media contained in 15ml centrifuge tube. After centrifugation at1200 rpm for 10 min, the cell pellet was resuspended in 1ml media, which was reseeded in the $25cm^2$ culturing flasks and incubated at 37^0C. The media was changed after 24 hrs byfollowing these steps.

3.2.1 Mitochondrial Isolation [Wieckowski et al., 2009]

Material: Cell's pellet, Phosphate buffer solution, homogenizing buffer, Refrigerated Centrifuge, flasks, eppendroffs.

Procedure: Cell pellets obtained from Buccal Cavity Cells was resuspended in 5ml of 1X PBS and centrifuged at 4^0C for 10 minutes at 1600 rpm. This was followed by sonication for 1-3 stocks of 5 seconds. After that centrifuged at 4^0C for 10 minutes at 5000rpm to remove broken cells. Then, transferred the supernatant in to a fresh eppendroff and again centrifuged after which the supernatant obtained was transferred in fresh eppendroff and centrifuged at 4^0C for 10 minutes at 12,000 rpm and the pellet was resuspended in 500µl homogenizing buffer.

3.2.2 Mitochondrial Transplantation [Chiron et al., 2007].

This experiment was done to see the efficiency of the cells by mitochondrial transplantation into cancer cells.

Material: Pellet of Mitochondrial, Cultured Cell lines, Mitotracker dye.

Procedure: The pellet of mitochondrial was put into mitotracker and then removed the supernatant and pellet resuspended in 10µl media. This medium was transferred

into 25cm^2 culturing flask already containing cultured cell which were incubated overnight. Next day the media was removed and the monolayer of cells was detached by trypsinization for 4 minutes. The effect of trypsin was inactivated by adding 1ml of media containing serum. Then 1ml media taken and added 500µl PBS in 35mm petri plate and the reading taken at 644/665nm (excitation/emission).

3.2.3 Evaluation of cell viability

3.2.3.1 MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] Assay [Baviskar et al., 2013].

It is a colorimetric assay, used to determine the viability of the cells which are metabolically active by measuring the reduction MTT by mitochondrial succinate dehydrogenase. The MTT gets reduced to an insoluble, colored (dark Purple) formazan product after entering into the cells and passes into the mitochondria, which get solubilized with an organic solvent like DMSO and can be measured multi-plate reader.

Material: MTT reagent, Phosphate buffer Solution, DMSO.

Procedure: The 96 wells plate was seeded with 100µl of media with cell of different ratio according to10, 000 cells and incubated for 24 hrs. Then cultured media were removed from the wells and the cells were washed with phosphate buffer solution (PBS). Then added 50µl MTT (5mg/ml) in each well and incubated the plate for 2-4 hrs. at 37^{0}C. After 2-4 hrs, discarded the MTT solution from each well and dissolved the intracellular precipitate in DMSO solution and placed in dark place for 20 minutes. After 20 min, the absorbance of the samples was measured at 570nm.

3.2.3.2 Dye Exclusion Assay [Mishell and Shiigi, 1980]

The dye exclusion assay is used to determine the number of viable cells present in a cell suspension

Material: 0.4% Trypan blue dye, Cell Suspension, PBS, Heamocytometer

Procedure: The Cell Suspension was centrifuged at 1200rpm for 5 min at 4^{0}C and then discards the supernatant and resuspends the pellet in 100µl PBS. Then the cells

were diluted with trypan blue dye in 1:1 ratio. Cell should be counted within 3-5 min of mixing with trypan blue dye. Then applied 1 drop of the diluted cells to a Heamocytometer and then count the stained (dead) and unstained (viable) cells separately, to obtain the total number of viable cells per ml. Then calculated the total number of viable cells by using formula= total number of cells (viable+dead) ×df (Dilution Factor).

3.2.4 Evaluation of Colony Forming Ability of Cells by CFU Assay [Clarke et al., 2006]

This assay was used to determine the colony formation activity of cells.

Material: Cells, 10X DMEM, FBS, Pen strip, 4% Agar.

Procedure: Added 1100µl autoclaved water in each well of the 6 well plate, followed by 200µl of 10X DMEM, 200µl FBS, 20µl penstripe and then 10,0000 cells were seeded and added 500µl agar in each well. Then incubated the plate for 10-20 days at 37^0C in CO_2 incubator. After 10-20 days count the colonies at colony counter.

3.2.5. Evaluation of ROS Production in the Cells by

3.2.5.1 H_2DCFDADye [Baviskar et al., 2013]

It is a chemically reduced form of fluorescein used as an indicator for reactive oxygen species (ROS) in cells

Material: H_2DCFDA Reagent, Phosphate buffer Solution, cultured cells.

Procedure: The 96 wells plate was seeded with 100µl of media with cell of different ratio according to10, 000 cells and incubated for 24 hrs. Then, added 50µl of H_2DCFDA in each well and incubated the plate for few minutes at 37^0C. After few min, the absorbance of the samples were measured at 485/530nm (excitation/emission).

3.2.5.2 Lipid Peroxidation (EC 1.11.1.11) [Devasagayam et al., 2003]

Lipid peroxidation is a well-established mechanism used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decomposes to form a complex series of compounds including reactive carbonyl compounds.

Material: 1N HCl, 50mM potassium phosphate buffer, pH- 7, MDA(Malondialdehyde Tetraethyl Alcohol), 0.375% TBA (Thiobarbituric Acid), 15% TCA (Tri Chloroacetic Acid), Cell Lysis Solution.

Procedure: To prepare the homogenized solution of cells, first took the already treated cells pellet and then homogenized the cells by adding 400µl of 1X PBS and centrifuged at 4^0C for 10 min at 3000 rpm. Then, 400 µl of supernatant of cells was taken in test tube and added 500µl of TCA and TBA and 1.6ml of Tris KCl in it. Then put the test tube into water bath for 1hrs at 80^0C. After an hr the test tube was put on ice and centrifuged at 4^0C for 10 min at 3000 rpm. Then, absorption was taken at 532nm in spectrophotometer.TBARS concentrations will be determined by comparison to an MDA standard curve following linear regression analysis (y = x (slope) + y int), express as MDA equivalents ([A 532] = a [MDA] + b) × df

Where: [MDA] is the µM concentration of MDA in the sample
A532 = Net absorbance at 532 nm of the sample
a = regression coefficient (slope)
b = intercept
df = dilution

3.2.6Assessment of Antioxidant Enzymes Activity in cells

3.2.8.2 SOD (Superoxide dismutase) Activity (EC 1.15.1.1) [Marklund and Marklund, 1974]

The antioxidant enzyme superoxide dismutase (SOD) catalyzes the conversion of superoxide anion radical ($O_2.^-$) to hydrogen peroxide and molecular oxygen. SOD helps prevent tissue damage by ($O_2.^-$).

$$2O_2^{-}\cdot + 2H^{-} \xrightarrow{SOD} H_2O_2 + O_2$$

Material: 6mM Pyrogallol, 6mM EDTA (Ethylene Diamine Tetra Acetic acid), 0.1 M Tris HCl Buffer, pH 8.2, 0.1ml Cell homogenate Solution.

Procedure: Prepared 0.1 M Tris HCl buffer of pH 8.2 and fresh 6mM pyrogallol solution. Then took the test tube and prepared the sample solution by adding1.5ml

Tris HCl buffer, 0.5ml of EDTA, 1ml of Pyrogalloland100µL sample. Then, absorption was taken at420nm after each interval of 30 sec. with respect to 1.5mlTris HCl buffer, 0.5ml of EDTA and 1ml of Pyrogallol as blank in spectrophotometer. The relationship between absorbance at 420nm and oxygen consumption was observed by this assay-

% inhibition = Control-Treatment ×100/Control =X

50% inhibition = X/50= Y (Unit/protein concentration in mg)

3.2.6.2 Catalase Activity (EC 1.11.1.6) [Chance and Maehly, 1955]

Catalase (hydrogen peroxide/hydrogen peroxide oxidoreductase), an important cellular antioxidant enzyme found in the peroxisomes of most aerobic cells, plays a very crucial role in defending our body against oxidative stress. It serves to protect the cell from toxic effects ensuing as a result of high concentrations of hydrogen peroxide (H_2O_2) by catalyzing its decomposition into molecular oxygen and water, without the production of free radicals.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_20 + O_2$$

Material: 50mM Sodium Phosphate buffer, 0.1 M Potassium phosphate, pH 7.0,360 µl H_2O_2 (Hydrogen peroxide) 30% in 0.1 M potassium phosphate, Triton-X,

Procedure: First prepared cultured cells homogenate solution (which was obtained by adding 2ml of triton-x in cells and then centrifuged at 4^0C for 10 min at 14,000 rpm).Then, prepared 0.1 M potassium phosphate buffer of pH-7 and fresh H_2O_2 solution with 0.1 M potassium phosphate buffer. Then taken the readings of 2900 µl H_2O_2 (light sensitive) by adding 100µL sample at 240nm after an interval of 30 sec. with respect to potassium phosphate buffer as blank in spectrophotometer.

Calculations used: Units/Protein concentration in mg = [(A/min (Blank) – A/min (Sample)]. d .1)/ V X 0.0436

Where, A/min = Change in Absorbance per min

d = dilution of original sample for Catalase Reaction

V = sample volume in Catalase Reaction, (x ml = 0.00x ml)

0.0436 = e^{mM} for hydrogen peroxide

1 = reaction volume in ml

3.2.6.3 Glutathione Reductase Activity (EC 1.6.4.2) [Sedlak and Lindsay, 1968]

Glutathione reductase catalyses the reduction of glutathione (GSSG) in presence of NADPH which is oxidized to $NADP^+$. The decrease in absorbance at 340nm is measured.

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP^+ + 2\ GSH$$

(GSH = Reduced Glutathione)

Material: 0.1 M Potassium Phosphate Buffer, pH 7.5, 0.2mM EDTA, 1.5mM $MgCl_2$, 0.5mM NADPH, 2mM Glutathione oxidized, Cell Lysis Solution.

Procedure: Prepared 0.1 M potassium phosphate buffer of pH-7 including PVP. Then taken reading of 200µl of potassium phosphate buffer including PVP, 100µl of EDTA, 100µl of $MgCl_2$, 200µl of NADPH, 200µl of Glutathione oxidized and 200µl of sample at 340nm after each interval of 30 sec. in spectrophotometer.

Calculations used: Units/Protein concentration in mg = $A/min/6.22x\ 10^{-3}$ .d

Where, A/min = Change in Absorbance per min

d = dilution of original sample for Catalase Reaction

$6.22x\ 10^{-3} = e^{mM}$ for NADPH

3.2.7 Mitochondrial membrane potential by JC-1 staining [Wong et al., 2002]

Mitochondrial membrane potential provides a valuable indicator of cells' health and functional status. The cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide) facilitates the discrimination of energized and deenergized mitochondria because the normally green fluorescent dye forms red fluorescent aggregates when concentrated in energized mitochondria in response to their higher membrane potential.

Material: JC-1 dye, Phosphate buffer Solution, cultured cells.

Procedure: The 96 wells plate was seeded with 100µl of media with cell of different ratio according to10, 000 cells and incubated for 24 hrs. Then added 50µl of JC dye (which was diluted in 200µl PBS) in each well and incubated the plate for a few

minutes at 37^0C. After few min, the absorbance of the samples was measured at excitation 490 nm and emission at 527 and 590 nm.

3.2.8 Evaluation of Drug Sensitivity in Cells

This experiment was done to see the sensitivity of transplanted cancer cells [Elliott et al., 2012].

Material: Doxorubicin Drug, Phosphate buffer Solution, cultured cells, MTT reagent, DMSO.

Procedure: The 96 wells plate was seeded with 100µl of media with cell of different ratio according to10, 000 cells and incubated for 24 hrs. Then added 10µl of 10mM Doxorubicin and incubated overnight. On next day, added 50µl of 5mg/ml with MTT in each well and incubated the plate for 2-4 hrs. at 37^0C. After 4hrs, discarded the MTT and washed with PBS. Then added 50µl of DMSO solution and placed in dark for 20 min. After 20 min, the absorbance of the samples was measured at 570nm.

CHAPTER- 4
RESULTS

4.1 Mitochondrial transplantation

In order to determine the extent and efficiency of mitochondrial transplantation into cancer cells, electrofusion and cell culturing techniques were used. In cell culturing, the mitotracker dye was employed and the cells cultured for 48hrs. At the end of 48hrs, the absorbance was read at a spectrophotometer at emission of 644nm and excitation of 665nm. Fig-4.1 represents the comparison between two techniques of mitochondrial transplantation, revealing that both techniques display similar results. Thus, it was concluded that mitochondria of the primary cells was entering the cancer cells with significant efficiency.

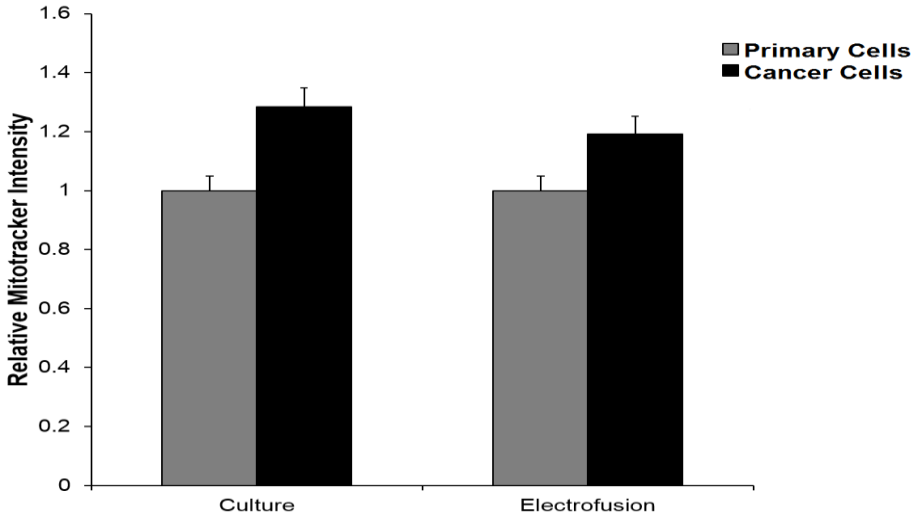


Fig- 4.1 Mitochondrial transplantation

4.2 Evaluation of Cell viability

4.2.1 MTT Assay

MTT assay, a safe and easy in vitro assay,was carried out in order to determine the cell viability as a measure of mitochondrial activity. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) is mainly a tetrazolium salt, employed in this colorimetric assay to determine the viability of the cells which are metabolically active by measuring the MTT reduction by mitochondrial succinate dehydrogenase. In active cells, MTT undergoes reduction to an insoluble, colored (dark Purple) formazan

product in response to the enzymes activity, which is then solubilized with an organic solvent like DMSO. The intensity of color, which is proportional to the cell viability [Meerloo et al., 2011] is measured spectrometrically at 570nm. In fig-4.2, the result shows the viability of HCT116 WT and HCT116 mutated p53. As compared to control the viability was found to be 85% in WT type and 50% in mutated p53 type due to presence of normal cell's mitochondrial with irrespective to the increased concentration of mitochondrial. In HCT116 p53 mutated type, the percentage of viable cells reduced due to mutation in p53 gene.

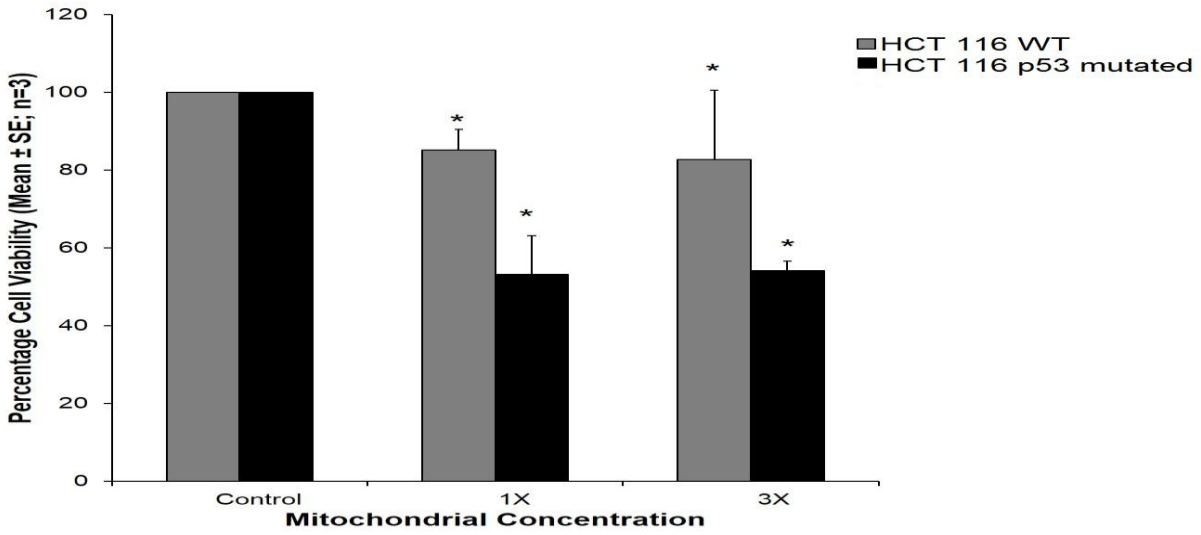


Fig-4.2 Percentage cell viability of HCT116 WT and p53 mutated cells in response to Primary cell Mitochondria treatment for 48 hrs. time period. Data is expressed as mean values ± S.E. (n=3) and had been analyzed by student t-test by followed different concentration of Mitochondrial and compared with respect to control. Statistically significant results were indicated by * with ($p < 0.05$) for HCT 116 WT and p53 mutated.

4.2.2 Dye Exclusion Assay

The dye exclusion Assay is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not [Strober, 2001]. Here, in this study Tryphan blue dye is used. According to this assay, a cell suspension is simply mixed with dye and then

visually examined to determine whether cells take up or exclude dye. In fig-4.3,the graph shows the result of cells viability in HCT116 WT and p53 mutated cell line. According to this result the control of both types have 100% viability of cells and as the primary cell's mitochondria transplant into both types of cell line, the viability of cells reduced. The viability of cells also depends upon the concentration of mitochondria, as the concentration of mitochondria increased by three folds; the viability of cells is reduced. Therefore, this is a good indication for this study since the mitochondria of the primary cell is stabilized in HCT116 cell line and shows their activity.

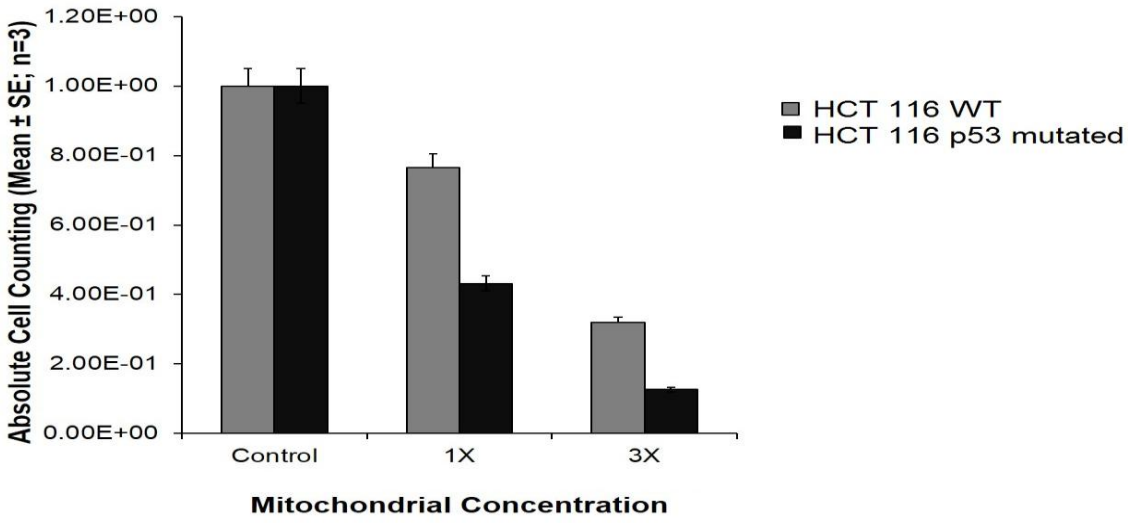


Fig-4.3Absolute cell counting of HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs.time period. Data is expressed as mean values ± S.E. (n=3).

4.2.3 Colony Forming Unit

The CFU (colony-forming unit) is the standard in vitro functional assay for measuring the number of colony forming cells. This assay is mainly used to determine the colony formation activity of cells. The CFU assay is commonly used to assess the colony forming capacity of a single cell which may reflect the cell's colonogenic (carcinogenic) potential. In this present study, the HCT116 cells were treated with primary cells mitochondria with different ratio, and to estimate the number of viable

cells in the culture this assay was done. In fig.4.4 the graph shows that the colony number decreased as the concentration of Mitochondria increased by 3 folds. The decrease in colony number reflects that transplanted mitochondria lead to decrease carcinogenic potential of these cancer cells.

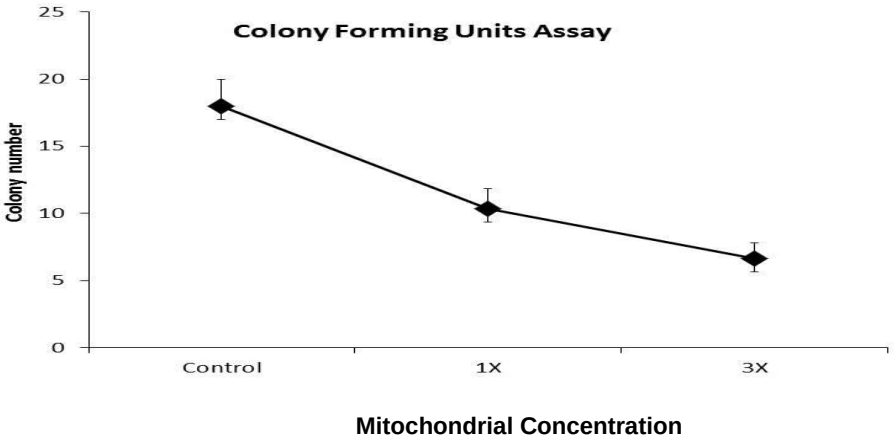


Fig-4.4 Colony numbers of HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 11 days. Data is expressed as mean values ± S.E. (n=3).

4.6 Assessment of ROS production in the cell

4.6.1 H_2DCFDA Assay

ROS (Reactive oxygen species)as a free radicals present in the cells but when these free radicals are produced in higher concentration, it caused imbalance in the cells which ultimately lead to cell damage and may initiate the process of carcinogenesis [Fuchs-Tarlovsky, 2013; Borges et al., 2013].The estimation of these intracellular ROS, following the oxidation of a non-fluorescent dyeH_2DCFDA(2',7'-dihydrodichlorofluorescein diacetate) to DCF(dichlorofluorescein) a fluorescent product by spectrophotometry. In fig 4.5, the graph shows the result of ROS concentration in the cells. According to result of this present study, in HCT116 WT the ROS concentration decreased and in HCT 116 mutated p53 the ROS concentration similar with respect to control. Therefore, it can be concluded that in HCT116 WT

ROS concentration decreased due to presence of mitochondria of the normal cells, as the concentration of mitochondria increased by three folds, the ROS concentration decreased further while in HCT116 mutated p53 the presence of primary cells mitochondria have no significant impact.

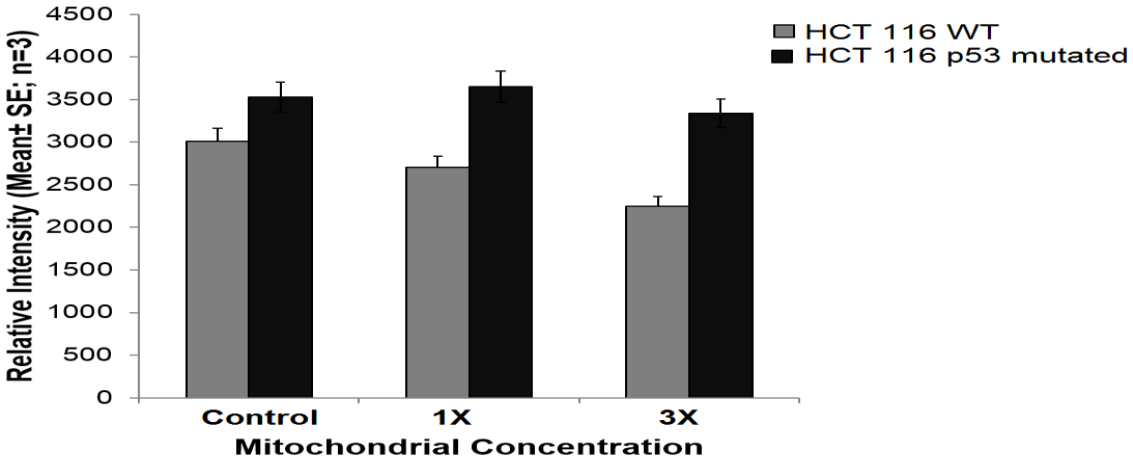


Fig-4.5 Relative intensity of HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

4.6.2 Lipid Peroxidation Assay

Lipid peroxidation is a well-established mechanism used as an indicator of oxidative stress in cells and tissues. The end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and the second one being known as "second messenger of free radicals" and it has numerous biological activities which resembling with reactive oxygen species. Certain diagnostic tests are available for the quantification of the end-products of lipid peroxidation. The most commonly used test is called a TBARS Assay (thiobarbituric acid reactive substances assay). Thiobarbituric acid reacts with malondialdehyde to yield a fluorescent product, which was absorbed at538nm in spectrophotometer. In this experiment, the HCT116 WT was treated with primary cells mitochondria. The graph (fig.4.6) represent that the lipid peroxidation was decreased due to presence of primary cells mitochondria. The decrease in Lipid peroxidation indicated that the

metabolic reprogramming of cancer cells increase due to introduction of healthy mitochondria.

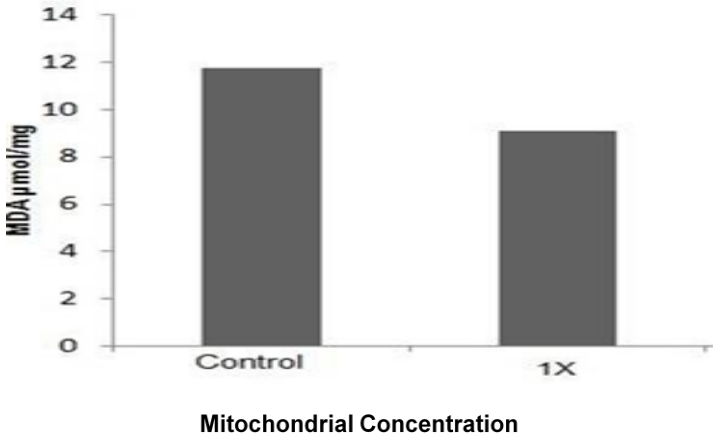


Fig-4.6 Concentration of MDA (µM/mg) in HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

4.7 Evaluation of Antioxidant Enzyme activity

4.7.1 Superoxide Dismutase Activity

Superoxide radicals are involved in many physiological and pathophysiological processes. They are produced as a by-product of respiratory electron transport and cytochome P450 reactions. The enzyme superoxide dismutase (SOD) decomposes superoxide anion into hydrogen peroxide and oxygen at a high reaction rate. Some literature highlighted that normal cell have more activity of SOD as compared to cancer cells [Oberley et al., 1979]. In this study the cells of HCT116 was treated with primary cells mitochondria. In fig.4.7 the graph shows that due to introduction of primary cells mitochondrial the SOD activity increased in cancer cell line. The increase in SOD enzyme activity indicated that the introduction of healthy mitochondria into cancer cells, leads to balance the oxidant and antioxidant system of cancer cells.

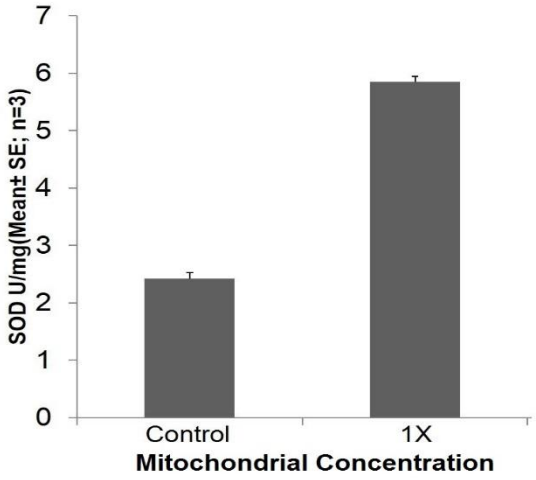


Fig- 4.7Concentration of Superoxide Dismutase (U/ protein in mg) in HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

4.7.2 Catalase Enzyme Activity

Catalase is a ubiquitous antioxidant enzyme, which protect the cell from its own production of toxic H_2O_2 (hydrogen peroxide). This oxidized enzyme break down the hydrogen peroxide into water and oxygen. Inhibition of catalase enzyme reduces the cellular ability to eliminate H_2O_2. Cancer cells have low level of catalase enzyme as compared to normal cells. As in this experiment, the HCT116 WT cell line was transplanted with primary cell mitochondria. In fig.4.8, the graph indicated that the catalase activity of HCT116 WT increased due to introduction of primary cells mitochondria. The increase in Catalase enzyme activity indicated that the introduction of healthy mitochondria into cancer cells, leads to balance the oxidant and antioxidant system of cancer cells.

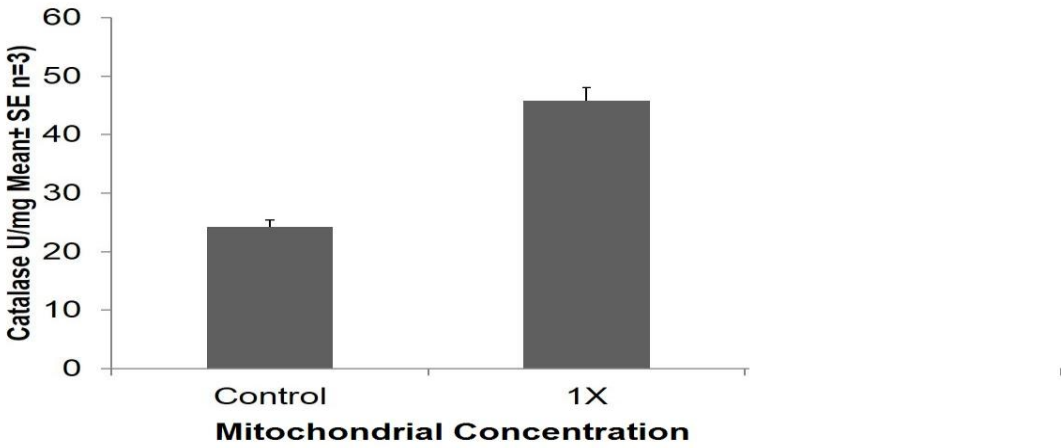


Fig- 4.8Concentration of Catalase (U/ protein in mg)inHCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

4.7.3. Glutathione Reductase Activity

Glutathione reductase plays an important role in protecting biological cell membranes against oxidative damage by increasing the level of reduced glutathione (GSSGR). In fig.4.9, the graph represented that glutathione reductase activity increased in HCT 116 WT cancer cell line due to introduction of primary cells mitochondria. Therefore, it can be concluded that the mitochondria of the primary cells are stabilized in HCT116 cell line and shows their activity against cells of HCT116. The increase in Glutathione Reductase enzyme activity indicated that the introduction of healthy mitochondria into cancer cells, leads to complete detoxification of ROS helps in balance the oxidant and antioxidant system of cancer cells.

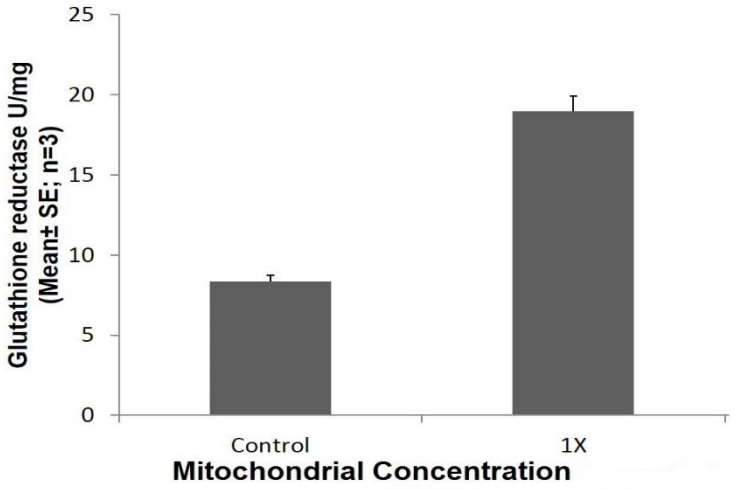


Fig- 4.9Concentration of Glutathione Reductase (U/ protein in mg)inHCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

4.4 Mitochondrial Membrane Potential by JC-1

Literature highlights mitochondrial membrane potential as an essential factor not only for mitochondrial function but also for beginning of apoptosis [Heiskanen et al., 1999;Jianget al., 2004].The membrane potential may range from high to low in healthy and apoptotic cells, respectively, therefore, indicating the mitochondrial dependent or independent cell death. Although various techniques are available for estimating the membrane potential, but most commonly used method is employing cationic dyes such as JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanineiodide). This dye facilitates the discrimination between the depolarized and polarized mitochondria. The dual fluorescence characteristics of the JC-1 can be used in the assessment by comparing the Relative Fluorescence Units (RFU) ratios of 590-600 nm (red)/ 527 nm (green). In the healthy cells, JC-1 forms complexes exhibiting red fluorescence as opposed to the green in apoptotic cells [Reers et al., 1991].When mitochondrial depolarization occurs, the red fluorescence decreases and the green fluorescence remains constant or increases, indicating cell death. In fig-4.10 the graph shows that as compared to control which having HCT-116 cell line only, in HCT-116WT type the membrane potential is high due to presence of primary

cell's mitochondria and in HCT-116 p53 mutated type the mitochondria are not polarized with irrespective to the concentration of mitochondria.

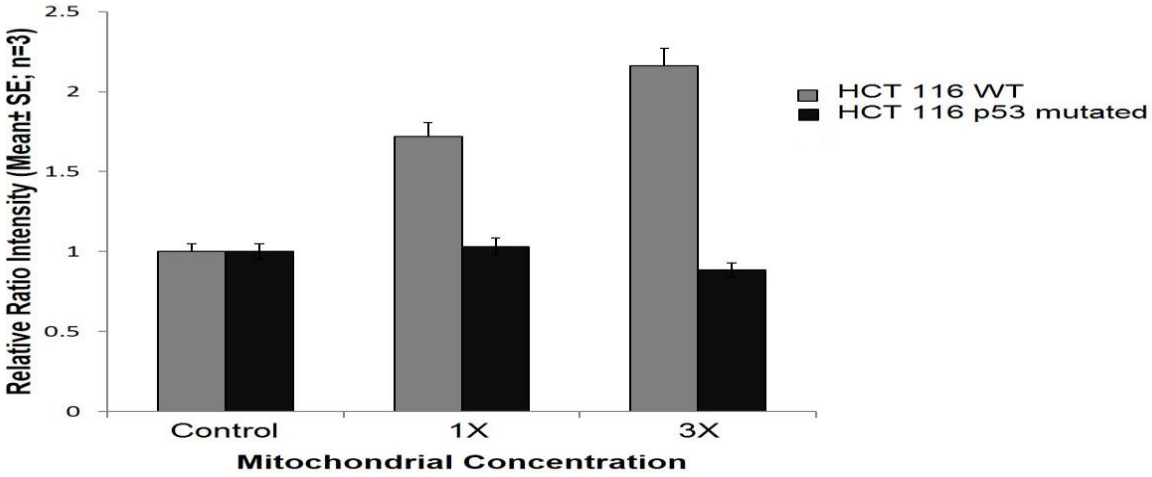
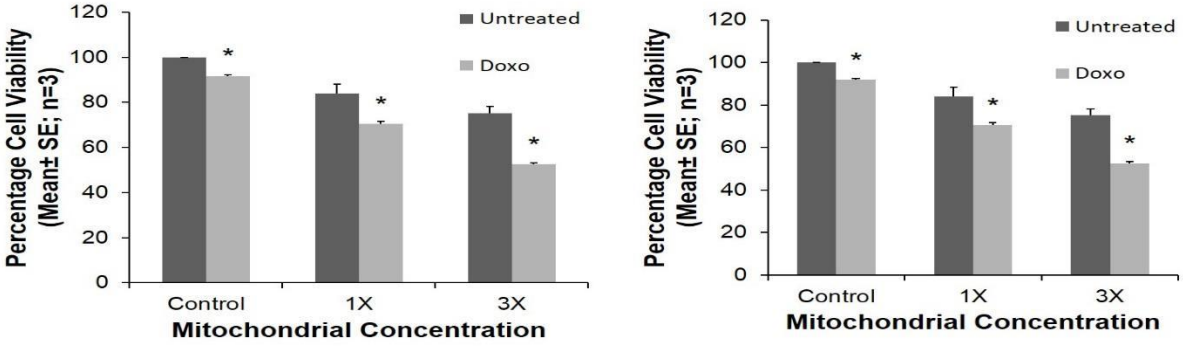


Fig-4.10 Relative ratio intensity of HCT116 WT and p53 mutated cells in response to primary cell mitochondria treatment for 24hrs. Data is expressed as mean values ± S.E. (n=3).

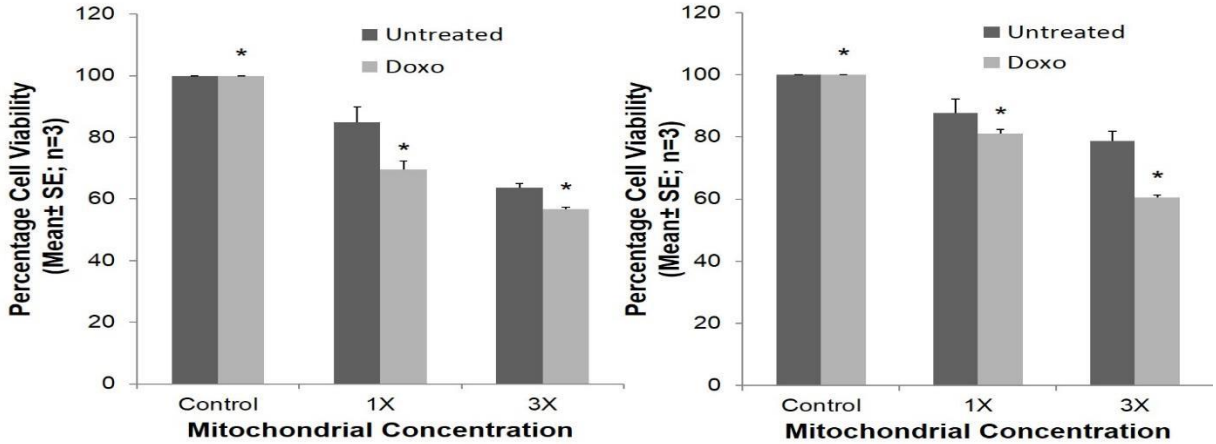
4.6 Evaluation of Drug Sensitivity in cells

Doxorubicin (DOX) is a chemotherapeutic drug belonging to the class of anthracyclines. It slows or stops the growth of cancer cells. DOX antitumor activity can be described to occur via two major mechanisms: one way being via blocking the enzyme topoisomerase II and other being the generation of free radicals, leading to DNA and cell membrane damage. Although, DOX is a valuable clinical antineoplastic agent, it has shown cardiotoxicity in addition to being promoting resistance to cancer cells [Kovalchuk et al., 2008: Tsang et al., 2003].Some literature highlighted that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients[Lai et al., 1991; Joly et al., 2002; Wallace et al., 2012].It has been shown that anticancer drugs like DOX also shows better effect on cells with less mutated mitochondria. In order to assess the direct role of mitochondria in DOX induced apoptosis, we treated the transplanted cells to sub- lethal dose of DOX and assess the percentage of cells undergoing apoptosis. Four cell lines HCT116 WT and p53 mutant (colon cancer), MCF-7 (breast cancer) and HepG2 (liver cancer) were transplanted with mitochondria isolated from healthy cells followed by 10µm of DOX

for 48 hours followed by MTT assay. The results show that all the cell lines showed increased cell death upon mitochondria transplantation in concentration (of mitochondria) dependent manner. The result thus show that all the cell lines showed increased cell death upon mitochondria transplantation in concentration (of mitochondria) dependent manner. The result thus shows that replacement of mitochondria of cancer cells increases their susceptibility towards anticancer drugs (Figure-4.11)



(a) HCT116WT(b) HCT116 P53 mutated



(c) MCF7(d) HepG2

Fig- 4.11Percentage cell viability of HCT116 (WT and p53 mutated), MCF7, HepG2 cells in response to Primary cell Mitochondria treatment for 48 hrs. time period toward

Doxorubicin. Data is expressed as mean values ± S.E. (n=3) and had been analyzed by student t-test by followed different concentration of Mitochondrial and compared with respect to control. Statistically significant results were indicated by * with ($p < 0.05$; comparison between DOX control and DOX 1× as well as 3×) for all the cell lines.

Chapter-5

DISCUSSION

Some literature highlighted that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients. This may result because of resistance to mitochondrial membrane permeabilization (MMP); [Lai et al., 1991; Joly et al., 2002; Wallace et al., 2012]. Therefore, the aim of my study is to check the mitochondrial mutations and their impact on carcinogenesis and drug sensitivity.

The mitochondria from buccal cavity cells had been isolated in small pellets. After the isolation of mitochondria, the mitochondria were transplanted into the cancer cells by using culture method. As previously reported that the membrane potentials of the cancerous cells, significantly lower as normal cells [Binggel et al., 1980]. Therefore, the transplantation of primary cells mitochondria into cancer cells become easy while the experimentation.

It was necessary to check the cell viability in cell suspension after the transplantation of mitochondria, which was done using, MTT Assay, Dye Exclusion Assay, Colony forming ability of cells by CFU Assay. By followed these experiments the results showed that the viability of cancer cells reduced due to presence of mitochondria from primary cells. After evaluated the cell viability, the mitochondrial membrane potential was checked by JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl carbocyanine iodide) Stain. The membrane potential may range from high to low in healthy and apoptotic cells, respectively, therefore, indicating the mitochondrial dependent or independent cell death [Kroemer et al., 2007]. From this experiment the It may be concluded that the mitochondrial membrane potential of HCT116 WT cancer cells was increased due to presence of mitochondria from primary cells. Therefore, there was no significant results in HCT116 p53 mutated cells as compared to HCT116 WT, may be due to mutation in p53 gene.

Mitochondria are the main source of oxygen metabolism, accounting for approximately 85–90% of the oxygen consumed by the cell. Mitochondria constantly metabolize oxygen by producing reactive oxygen species (ROS) as a byproduct. These organelles have their own ROS scavenging mechanisms that are required for cell survival [Chance et al., 1979; Shigenaga et al., 1994; Kirkinezos and Moraes, 2001].The H_2DCFDA Assay was performed to assess the ROS production in the cells. The results showed that the ROS production of HCT116 WT cancer cells was reduced due to presence of mitochondria from primary cells, which indicated that, the primary cells mitochondria is stabilized in HCT116 cell line and shows their activity against cells of HCT116WT. Furthermore, there was no significant results in HCT116 p53 mutated cells as compared to HCT116 WT.

Lipid peroxidation is a natural process which generated in small amounts in the body, mainly by the effect of several reactive oxygen species (hydroxyl radical, hydrogen peroxide etc.).These reactive oxygen species readily attack the polyunsaturated fatty acids of the membrane lipids and dangerous for the viability of cells, even tissues [Mylonas and Kouretas, 1998]. In this experiment, the HCT116 WT cells were treated with primary cell mitochondria, and the result shows that the lipid peroxidation decreased due to presence of primary cells mitochondria. So, the mitochondria of primary cells may have higher membrane potential, which helps to reduce the lipid peroxidation process in the cells.

Mitochondria have their own ROS scavenging mechanisms, and because it produce higher rate of ROS than their scavenging capacity, resulting in the incomplete metabolism of approximately 1–3% of the consumed oxygen [Boveris and Chance, 1973;Nohl and Hegner, 1978]. The byproducts of incomplete oxygen metabolism are superoxide ($O_2.^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•), which produced by transfer of a free electron to molecular oxygen. Then, the mitochondrial enzymes scavenged the ROS by different process [Turrens and Boveris, 1980]. So, the antioxidant enzyme activity of the cells by different parameters such as Catalase, SOD (Superoxide dismutase) and Glutathione Reductase was evaluated.

The Catalase enzyme activity was increased due to the presence of primary cells mitochondria, which indicated that due to presence of healthy mitochondria the ROS production in the cells was reduced, and which may be lead to balance between oxidant and antioxidant system of the cells. The SOD enzyme activity was increased due to presence of primary cells mitochondria, which indicated that mitochondrial enzyme manganese superoxide dismutase (MnSOD) react with ROS, which produced by HCT116 WT cancer cell lines to H_2O_2 and balance the oxidant and antioxidant system of the cells. Similar in Glutathione Reductase, the enzyme activity was increased due to presence of primary cells mitochondria, which indicated that mitochondrial enzyme Glutathione reductase (GSPx) reduced glutathione (GSH) as a coenzyme and converts H_2O_2 to water, which was produced by enzyme manganese superoxide dismutase (MnSOD). Therefore, completely detoxification of ROS helps in balance the oxidant and antioxidant system of the cells.

Some studies show that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients [Lai et al., 1991; Joly et al., 2002]. In this study, the drug sensitivity of cancer cells was evaluated by using anticancer drug DOX (Doxorubicin). In this experiment the cells was treated with primary cells mitochondria and due to presence of primary cells mitochondria, the sensitivity of doxorubicin was enhanced in all cell lines HCT116WT, HCT116p53mutated, BCF7and HepG2 .

Overall it may be concluded that replacing cancer cell mitochondria may lead to decrease cell survival; colonogenic capacity of these cells. These changes may be due to metabolic reprogramming of cancer cells as indicated by various free radicals and antioxidant enzymes assays. Furthermore, replacing mitochondria cause increase sensitivity of cancer cells against chemo resistance.

Thus futuristic therapies may be designed to replace cancer cell mitochondria but the challenge of working at tissue level remains. Once this technical issue is solved, mitochondrial replacement therapies may become potent for cancer and various other mitochondrial diseases.

SUMMARY

Mitochondria, the power house of the cell, are surrounded by two membranes, and have their own genome. Mitochondria are main sources of energy, which responsible for creating more than 90% of the energy needed by the body to sustain life and support growth. In case of any dysfunction of mitochondria less energy is generated within the cells, which lead to cell injury and even cell death follow. The metabolic shift in the mitochondrial genome also may due to mutations, which may either inherited or spontaneous in nature. Mutations in mtDNA are the result of mitochondrial diseases such as Cancer.

Some previous study suggests that, although mutations in mitochondrial genes are common in cancer cells, they do not inactivate mitochondrial energy metabolism, but rather alter the mitochondrial bioenergetics and biosynthetic state [Wallace, 2012]. Some studies also highlighted that owing to mitochondrial dysfunction the clinical trial of many anticancer drugs has failed in patients [Lai et al., 1991; Joly et al., 2002].

To counteract this problem in the present study, I evaluated the impact of mitochondrial transplantation on cancer cells. After the introduction of mitochondria from normal cells to cancer cells, we checked the changes in metabolism by monitoring and comparing the different parameters i.e. Cell viability, mitochondrial membrane potential, ROS production, Antioxidant enzymes activity and their drug sensitivity.

It can be summarized from the results that the healthy mitochondria transplanted to cancer cells decrease carcinogenesis and have drug sensitivity. The present study revealed the some important conclusions as described below:

- This study concluded that the healthy mitochondria transplanted to cancer cells decrease carcinogenesis. So, it may be used as futuristic cancer remedy.
- The antioxidant system of normal cells mitochondria are better than cancer cells mitochondria, which may inhibits the cancer cells proliferation.

- Transplanted mitochondria also have increased drug sensitivity. Therefore, Mitochondria may be as possible target for cancer therapeutics.

Therefore, further strategies may be designed to rectify the mitochondrial metabolism, and to better understand the various cellular and metabolic pathways, impacted by Mitochondria transplantation which may have potential for other disease therapy. Furthermore, potent delivery vehicle (nanoparticles) may be designed to rectify the mitochondrial malfunctioning.

References

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