TO STUDY THE EFFECT OF INSULIN ON ADVANCED ANDROGEN-INDEPENDENT PROSTATE CANCER (PC-3) CELLS

A Dissertation submitted to the Central University of Punjab

For the award of

Master of Philosophy

In

Biosciences

BY
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August 2012

CERTIFICATE

I declare that the thesis/dissertation entitled "**To Study The Effect of Insulin on Advanced Androgen-Independent Prostate Cancer (PC-3) Cells**" has been prepared by me under the guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, 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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ABSTRACT

To Study The Effect of Insulin on Advanced Androgen-Independent Prostate Cancer (PC-3) Cells

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Key words (Five minimum):	Insulin, Cancer cell line (PC-3), Oxidative stress, SOD (Superoxide dismutase), Matrix metalloproteinases.

Prostate cancer is one of the major causes of mortality in males over the age of fifty all over the world. Many factors including genetics and diet have been associated with the development of prostate cancer. Hyperinsulinemia has been found to be associated with higher risk of Prostate cancer. Diabetes type-2 is accompanied with hyperinsulinemic state. Both cancer and diabetes are metabolic disorders and often diabetes is correlated with cancer. This study reveals that insulin acts as a mitogen hence increases proliferation in PC-3 cells. Reactive oxygen species are by product of cellular metabolism. Insulin treatment increases cellular metabolism due to which ROS level also increases at higher insulin doses. ROS is necessary for many cells signalling process, abnormal increase in ROS level can cause mutational DNA damage and affects protein folding. Antioxidants and free radical balance is critical for normal cellular functioning. Superoxide dismutase is an important antioxidant enzyme, which keeps ROS level low by dismutation of superoxide anion into hydrogen peroxide. This is further metabolised by catalase. In our study we have found that at lower insulin doses SOD level increases but at higher insulin doses SOD expression decreases significantly. This may be the possible reason of ROS increase. Matrix metalloproteinase's expression is modulated by insulin, which can lead to increase in malignancy. All factors stated above indicate that hyperinsulinemia can lead to tumor progression.

(Abhimanyu Kumar) (Dr. Sanjeev Thakur)

ACKNOWLEDGEMENT

Although only one name is on the cover of this M.Phil dissertation, but actually there are many hidden names that helped me to make contents in between the covers. I take this opportunity to express my thankfulness to all of them.

Although I did not find words that could express my thanks to a comparable extent for the help and support that he provided me, but still trying to thank my guide **Dr. Sanjeev Thakur,** who guided my M.Phil. and provided all the facilities to complete my dissertation. He has motivated me very much and this M.Phil. work would not have been completed without his help. I thank you sir, for all the help and advice (both personal as well as professional).

My special thanks to **Dr. Sandeep Singh** who helped me in my work. I thank you sir, for you prosperous guidance during practical and thesis writing work. I would like to thank Dr. Pankaj Bhardwaj, Dr. Felix Bast, and Dr. Anjali Singh for their guidance and help during my M.Phil. degree and experimental work.

I would also like to thankfully acknowledge Dr. R.G. Saini, Coordinator of Centre, Centre for Biosciences and Dr. P. Ramarao, Dean, Academic affairs for their valuable guidance and Dr. Jai Rup Singh, Vice Chancellor, Central University of Punjab, Bathinda for providing me research facilities to carry out my work.

I am also thankful to Dr. Saranpal MD, Biochemistry Gee Ess diagnostic laboratory Bhatinda for helping me for ELISA Microplate reader analysis at their laboratory.

I am extremely thankful to all my friends in CUP, Bathinda, Rajeev Mann Devendra Gahlot, Bibekananda Sarkar, Ravi Cholia, Md. Zahid, Shweta and Seema S. for their cooperation, guidance, support and help during M.Phil. Iam also thankful to all seniors and juniors for their support and help during M.Phil. degree.

I am thankful to Gopal Soni, Ashwani Kumar for their help and assistance in lab during experimental work. It was not possible for me to complete my work without the love, care, understanding and support of my parents and family members who were always in front of me to extend their support at times of desperation and shaping my dissertation. They stood by me at most difficult times and their emotional warmth and tenderness was unparallel. I would like to thankfully acknowledge Central University of Punjab, Bathinda, for providing me Fellowship. Finally thanks to almighty God.

Abhimanyu Kumar

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LIST OF ABBREVIATIONS

S. No.	Abbreviation	Full Form
1.	ATP	Adenosine triphosphate
2.	DMEM	Dulbecco's modified eagles medium
3.	DMSO	Dimethyl sulfoxide
4.	dNTP	Deoxynucleotide triphosphates
5.	DCFDA	Dichlorofluorescein diacetate
6.	DDC	Diethyldithiocarbamate
7.	DNA	Deoxyribonucleic acid
8.	ECM	Extracellular matrix
9.	HCl	Hydrochloric acid
10.	ICAM	Intercellular cell adhesion molecules
11.	IGF-IR	Insulin like growth factor receptor
12.	IR	Insulin receptor
13.	IRS	Insulin receptor substrate
14.	MMP	Matrix metalloproteinase
15.	mRNA	Messenger ribonucleic acid
16.	MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
17.	NCRP	National cancer registry programme
18.	nM	Nano molar
19.	PBS	Phosphate buffered saline
20.	PBCR	Population based cancer registry programme
21.	PI3K	Phosphatidylinositol-3-kinase/Phosphoinositide-3-kinase
22.	PDK	Phosphoinositide dependent kinase
23.	PCR	Polymerase chain reaction
24.	PIP3	Phoshatidylinositol-3-phosphate
25.	PTP	Protein tyrosine phosphatases
26.	RNA	Ribonucleic acid
27.	SH2	Src homology-2
28.	ROS	Reactive oxygen species
29.	SOD	Super oxide dismutase
30.	SDS	Sodium dodecylsulfate
31.	VEGF	Vascular endothelial growth factor
32.	WHO	World health organization
33.	~	Approximately
34.	µL	Microlitre

Chapter I

Introduction

1. Introduction

Cancer is one of the leading diseases responsible for high mortality rates worldwide. It is a multi-factorial group of diseases involving uncontrolled growth of cells resulting in formation of tumors. Malignant tumor can metastasize into surrounding tissues as well as in distant organs (Stratton et al., 2009). All cancers arise due to the change in DNA sequence of cancer cell genome. It involves over hundred distinct diseases with variety of risk factors and different epidemiology (Stratton, et al., 2009). Cancer is similar to an evolutionary process in which cells acquire mutations randomly and are selected based on the survivability. Cells acquainted with deleterious mutations are eliminated, while occasionally cells acquire advantageous mutations that allow uncontrolled proliferation and metastasis. Cancer cells have the trait to proliferate indefinitely and it is achieved through the release of mitogenic signals by themselves or inducing surrounding cells to release mitogenic signals also termed as paracrine signalling.

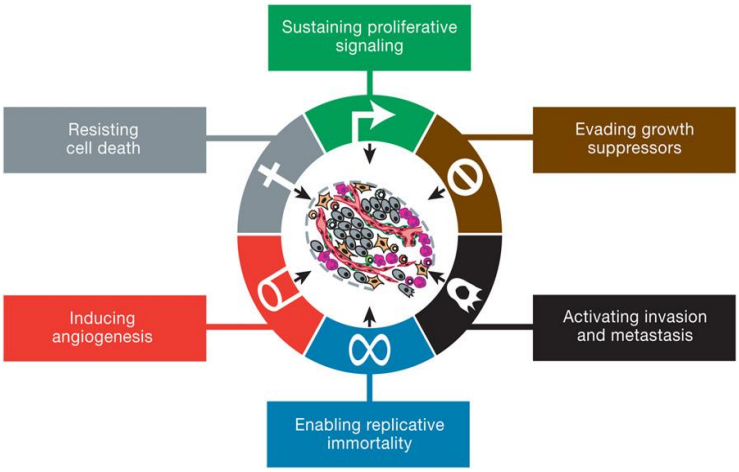

***Figure1.1*:** Major hallmarks of cancer (Hanahan & Weinberg, 2011)

1.1 Prostate cancer

Prostate cancer is the major non cutaneous cancer diagnosed in the males in United States (Bannuru et al., 2011). Prostate gland is found in men and is located in front of the rectum under the bladder. Prostate contains many small

glands which make about twenty percent of the fluid constituting semen. Prostate cancer tends to develop in men over the age of fifty and this is one of the most prevalent types of cancer in men.Prostate cancer start in the prostate cells and can invade and damage normal tissues. Many factors, including genetics and diet have been found to be associated in the development of prostate cancer. Prostate cancer incidence has increased and mortality has decreased due to the early detection and introduction of blood based prostate specific antigen test (Wilt et al., 2008). Hyperinsulinemia has also been found to be associated with higher risk of prostate cancer (Giovannucci, 2005; Kaaks & Stattin, 2010). The presence of prostate cancer may be indicated by symptoms, physical examination and prostate-specific antigen (PSA), or biopsy. Common treatments for prostate cancer include external beam therapy, interstitial radiation therapy, surgery to remove gland and androgen deprivation therapy (Wilt, et al., 2008). Men with prostate cancer and increased body mass index tend to have lower PSA level as compared to thinner men with similar cancers. So this can delay prostate biopsy recommendation in obese persons and result in increased risk of higher grade cancer (Ramahi et al., 2012). Prostate cancer cell lines are used to investigate the mechanism involved in the progression of prostate cancer. LNCaP, PC-3 and DU-145 are most commonly used prostate cancer cell lines.

1.2 Diabetes and Cancer

Cancer is characterized by uncontrolled cell division and diabetes is a metabolic disorder characterized by hyperglycaemia. Both the diseases involve external as well as internal risk factors. Type-1 diabetes is characterised by hyperglycaemia, endogenous deficiency of insulin secretion and requires administration of exogenous insulin (Vigneri et al., 2009). Type 2 diabetes shows hyperinsulinemia, hyperglycaemia and hyperlipidemia for prolonged time (Novosyadlyy et al., 2010). In type-2 diabetes exogenous insulin is required only when β cell function fails. Diabetes is a complex disease in which different factors interact to alter insulin action, secretion and resulting in hyperglycaemia (Kulkarni et al., 2003). Interaction between Prostate cancer and diabetes can be explained on the basis of hyperinsulinemia and hyperglycaemia conditions that may lead to increase in

tumor proliferation and metastasis (Snyder et al., 2009). Insulin acts as a growth factor for prostate epithelial cells. Beside insulin, insulin like growth factor IGF-1 and IGF binding protein-3 have also been found to be associated with prostate growth (Snyder, et al., 2009). Growth promoting effect of hyperinsulinemia is mediated by the activation of insulin receptor (IR), insulin like growth factor receptor (IGF-IR) and phosphoinositide 3 kinase (PI3K) pathway (Novosyadlyy, et al., 2010). Insulin receptor expression increases in breast cancer tissue as compared to normal breast tissue. Insulin pre-eminent role is metabolic but it also has mitogenic effects in the malignant cells at receptor and post receptor level. Hyperglycaemia, oxidative stress and obesity results in increased risk of cancer in diabetes (Vigneri, et al., 2009). Cancer is associated with higher mortality in diabetic patients as compared to non diabetic. Insulin analogues are used in the treatment of diabetes mellitus (Weinstein et al., 2009). Insulin B10ASP was the first insulin analogue to be developed with single amino acid substitution. It shows tenfold increase in mitogenicity as compared to human insulin. Alteration in the structure of insulin increases its mitogenic effects as demonstrated by enhanced DNA synthesis and cell division. This mitogenic effect is due to the prolonged binding to the insulin receptor and cross reactivity with IGF-1 receptor (Smith & Gale, 2009). Long acting insulin analogue glargine, detemir and short acting analogues lispro, aspart exhibits activity like IGF-1 in the cultured cancer cells. These insulin analogues exhibit in vitro proliferative and antiapoptotic activities in various cancer cell lines as compared to insulin (Weinstein, et al., 2009). Molecular mechanisms linking diabetes and cancer are multi-factorial and require extensive and better designed study.

CHAPTER-II

Review of Literature

2. Review of Literature

2.1 Cancer statistics

2.1.1 World scenario

Cancer is a major cause of death in developed countries also it is the second leading cause of death in the developing countries (Jemal et al., 2011).Cancer is responsible for one in eight death worldwide (Stratton, et al., 2009). Prostate cancer is second most frequently diagnosed cancer and the sixth leading cause of cancer deaths in males, accounting for 14% (903,500) of the total new cancer cases and 6% (258,400) of the total cancer deaths in males in 2008. Incidence rates vary by more than 25-fold worldwide, with the highest rates recorded primarily in the developed countries of Oceania, Europe, and North America largely. In contrast, males of African descent in the Caribbean region have the highest prostate cancer mortality rates in the World, which is thought to reflect partly difference in genetic susceptibility (Jemal, et al., 2011). American cancer society estimates that in 2009 approximately 192000 men were diagnosed with prostate cancer and 27000 patients died of this disease (Bannuru, et al., 2011).

Table 2.1: Incidence Rates of Prostate Cancer by Race

Race/Ethnicity	Male
All Races	154.8 per 100,000 men
White	146.9 per 100,000 men
Black	236 per 100,000 men
Asian/Pacific Islander	85.4 per 100,000 men
Indian/Alaska Native	78.4 per 100,000 men

Source: National Cancer Institute (SEER 2009)

2.1.2 Indian scenario

Cancer is the second most common disease in India causing maximum mortality and accounts for 0.3 millions death every year (Ali et al., 2011). Cancer prevalence in India is estimated to be 2 to 2.5 million with 7-9 lakh new cases being detected every year(National Cancer Control Programme 2005). Purpose of population based cancer registry programme (PBCRs) is to determine the rate of cancer incidence in different regions and in different subgroups of population in India. Cancer incidences are new cases diagnosed in a population in a specified time period. Therefore, Indian council of medical research has started national cancer registry programme (NCRP) since 1982. Broad purpose for the initiation of this project is to develop atlas of India regarding cancer incidence. It has been estimated that current probability of developing cancer in India form 35-64 years is 4.67% in males and 6.55% in females, life time risk was found to be 9.05% and 10.2% respectively. According to these estimates 1 in 8 men and 1 in 10 women in India can expect to develop cancer after age of 35. In women risk of developing uterine cervix and breast cancer is very high (Murthy et al., 2011). Reasons for this high prevalence of cancer in India can be attributed to internal (genetic mutations, hormonal) and external factors (environmental, food habits and industrialization). Economic loss has also increased with the increasing cancer patients. In 2004 numbers of cancer patients were 819354 and it accounted for loss of 25.16 million US dollars, which increased in 2009 to 962832 patients and total economic loss was 274.10 US million dollars (Ali, et al., 2011) .

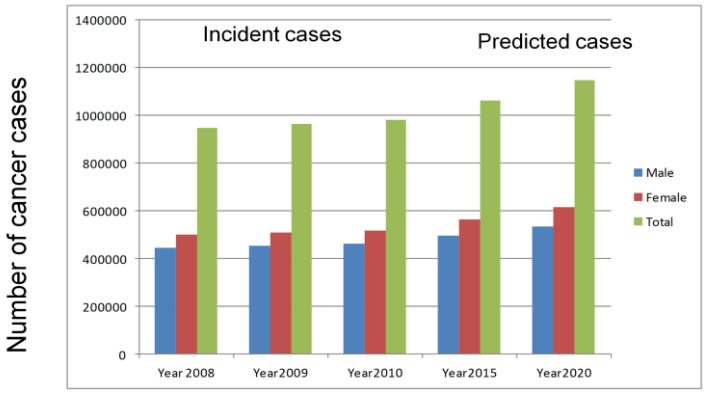

Figure* 2.1:** Cancer incidence in India ***Source: (ICMR February 2010)

Table 2.2: Total number of cancer cases registered in all PBCRs

Registry	Male	Female	Total Cases
Bangalore(2006-07)	5645	6979	12624
Barshi Rural (2006-08)	389	394	783
Barshi Expanded(2007-2008)	1548	1977	3525
Bhopal(2006-2008)	2015	1962	3977
Chennai(2006-2008)	7392	7866	15258
Delhi(2006-2007)	13720	12613	26333
Mumbai(2006-2008)	15745	17485	33230
Cachar district(2007-2008)	1586	953	2539
Dibrugarh(2006-2008)	1483	1038	2521
Kamrup urban district(2006-2008)	1952	1304	3256
Manipur State(2006-2008)	1934	2035	3969
Imphal west district(2006-2008)	605	752	1357
MR Excl Imphal west (2006-2008)	1329	1283	2612
Mizoram State(2006-2008)	1838	1542	3380
Aizwal district(2006-2008)	914	778	1692
MZ Excl Aizwal	924	764	1688
Sikkim state(2006-2008)	606	549	1155
Ahemdabad (rural) (2006-2008)	1400	1002	2402
Ahemdabad (urban) (2006-2008)	5215	4227	9442
Aurangabad(2005-2008)	928	960	1888
Kolkata(2006-2007)	4611	4427	9038
Kollam(2006-2008)	4656	4374	9030
Nagpur(2006-2008)	2668	2754	5422
Pune(2006-2008)	4047	4547	8594
Thiruvananthapuram (2005-2008)	2851	2904	5755

Source: National Cancer Registry Programme (2006-2008)

2.1.3 Cancer incidence in Punjab

Green revolution and commercialization of agriculture in Punjab has resulted in the irreversible and long term health repercussions. Indigenous agriculture was replaced by modern agriculture and indigenous seed varieties were replaced with high yielding crops. This also led to soil contamination due to uncontrolled use of pesticides and chemical fertilizers for increase in crop production. This change has resulted in health problems as rise in cancer cases, reproductive health problems and kidney ailments (Yeole, 2008). Pesticide exposure is found in farmers and in the people living in the nearby areas of heavily treated land. Adverse effects of pesticide are linked with the diseases like cancer, kidney failure, infertility and nervous disorders (Singh & Kaur, 2012). In some parts of Punjab uranium has also been found at alarmingly higher levels. In Bathinda 50% of subsurface water samples have shown higher uranium concentration (>60µg/ml permissible limit by Atomic energy regulatory board) (Thakur et al., 2008). Uranium can pose both radiological and chemical problems. It can reach in the human body through food chain and accumulate in the vital organs causing radiological damage. Uranium content was found to vary from 0.38mBq/g in mustard to 4.60 mBq/g in wheat (Samples of Bathinda district). While in case of milk the uranium content was found to vary from 28.57 to 213.36 mBq/ (M. Kumar et al., 2009).

Table2.3: Distribution of cancer cases in Malwa region of Punjab state (2005)

S. NO.	District	Population	No of cancer patients	Cancer patients per lakh population
1	Muktsar	827906	453	54.7
2	Bathinda	1200736	711	59.2
3	Faridkot	585500	164	28.0
4	Mansa	731535	420	57.4

Source: (National Cancer control Programme 2005)

2.2 Cancer correlation with diabetes

Epidemiological studies have shown higher risk of cancer in diabetes type 2 and obesity, which is attributed to hyperinsulinemia and insulin resistance conditions prevailing in these diseases. Cancer prevention studies suggest that 14% death in men and 20% death in women are related to obesity. Obesity results in insulin resistance and that further culminates in to hyperinsulinemia. Higher insulin levels are accompanied with enhanced IGF-I levels. Crosstalk between IGF-I, IGF-II and insulin signaling can increase the mitogenic effect and hence cancer risk (Gallagher & LeRoith, 2010). Insulin receptor isoform IR-A over expression in cancerous tissues and in cancer cell line leads to IGF-II signaling through insulin receptors (Vella et al., 2002).

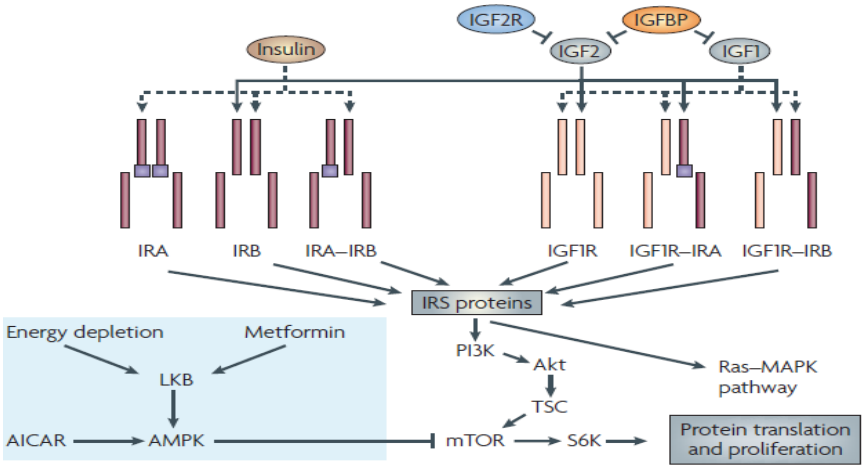

Figure-2.2 Crosstalk between insulin and IGF signaling (Pollak, 2008)

Experimental studies have shown dose dependent relationship between glucose concentration and tumor growth. However cancer cells have higher uptake level of glucose and can complete their requirement in the normal glycaemic conditions. If hyerglycaemia is link between diabetes and cancer (as hyerglycaemia is found in diabetes), then decrease in glucose level by insulin should also decrease cancer risk. But cancers are heterogeneous, so response to insulin will also be varying and response is not of universal nature (Johnson & Pollak, 2010).

2.3 Role of Insulin in cancer

Insulin is secreted by pancreatic β cells and it plays a major role in glucose homeostasis (B. Ahmad, 2004). Insulin molecule consists of two peptide chains (A, B) and containing 21 and 30 amino acids, respectively. Two chains of insulin are derived from the single chain precursor was demonstrated be Steiner et al in 1967. Intrachain disulfide bond is found between amino acid 6 and 11 in A peptide chain, while in B peptide chain interchain disulfide bond is found in amino acid (7-7) and (20-19) (B. Ahmad, 2004). Insulin occurs in two forms monomeric and hexameric form. In the circulation insulin occurs in monomeric, while hexameric form is found in crystal and in β cell granules.

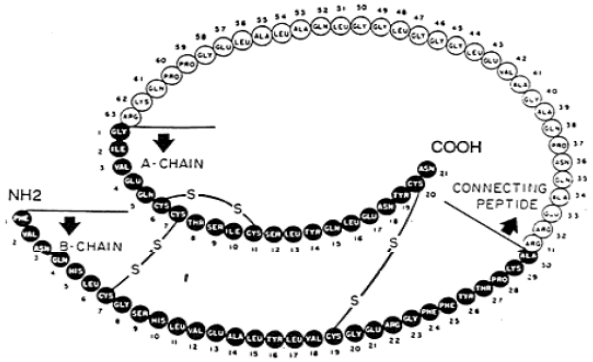

Figure 2.3: Human Insulin structure ***Source:*** (Diabetesmanager 2009)

In the endoplasmic reticulum proinsulin is synthesized on membrane bound polysomes. Conversion of proinsulin into insulin takes place in membrane bound vesicle either in endoplasmic reticulum or in Golgi apparatus. Proinsulin is converted into insulin connecting peptide (C peptide) and stored in the vesicles until they are released into the blood (Permutt et al., 1984). Insulin is secreted in response to higher blood glucose level. Insulin binding with its receptor leads to the localization of glucose transporters on the cell membrane, which was initially found in the vesicles inside cells (Albanes et al., 2009). Beside this insulin has effect on the gluconeogenesis, activation of transcription factors and protein synthesis. Insulin increases uptake of glucose and fatty acid synthesis in prostate cells also. Beside this insulin has antiapoptotic property. In diabetes mellitus -2 high serum concentration of insulin is found. Abnormality in the interaction

between insulin and its receptor can lead to insulin resistance condition. Insulin has potent mitogenic and growth stimulatory effects on prostate. Higher serum concentrations have been found to be associated with the risk of developing Prostate cancer. In 1970s insulin was first reported to induce growth of mammary tumors in mice (Gallagher & LeRoith, 2010). Studies have provided evidence that patients with higher serum levels of insulin or c-peptide (an analyte highly correlated with insulin) are at increased risk of prostate cancer. The risk of prostate cancer related death among men diagnosed with prostate cancer was 2.7-fold higher in men in the top quartile of c-peptide as compared to men in the bottom quartile (Cox et al., 2009). Exact mechanisms underlying the increased risk of cancer in hyperinsulinemia is unknown, but it may involve the influence of hyperinsulinemia in enhancing free or bio available IGF-I levels (Giovannucci, 2005). Hyperinsulinemia is associated with increased AKT activation and aggressive tumor behaviour in a prostate cancer (Cox, et al., 2009).This is a well known fact since long that glucose is required for tumor growth and high insulin levels are related to metastasis. Warburg first time in 1906 showed that the metabolism in cancer cells generate lactic acid (lactate) as glucose metabolism is anaerobic rather than aerobic. Permanent increase in anaerobic glucose utilisation by primary tumor transforms it to aggressive type. High level of lactate is associated with increase in the acidity around the tumor. Increase in acidity is however beneficial for the cancer cells. However cancer cells are resistant because of increased H^+ transporter activity. Growing cancer cells in hyperglycaemic and diabetic (high insulin) conditions alter the expression of genes involved in cell cycle, cell migration and cell-cell adhesion regulation.

2.3.1 Insulin Receptors

Most reports of insulin receptor (IR) expression concern tissues that are classically insulin-responsive, such as liver, muscle and fat. In obese cancer patients (cancer patients who are normal weight but metabolically obese) insulin resistance present in classic insulin-responsive organs such as liver, fat, and muscle leads to hyperinsulinemia, which may lead to aggressive behaviour of those cancers that are IR (insulin receptor) positive and insulin sensitive (Giovannucci, 2005). Both IRs and IGF-IRs exist as tetrameric complexes comprised of two half receptors, each of which is comprised of an alpha chain and its beta chain. It is now

recognized that in cells that express both IRs and IGF-IR receptors can also form hybrid receptors. These consist of a half insulin hybrid receptor (insulin receptor alpha chain and insulin receptor beta chain) complexed with a half IGF-I receptor (IGF-I receptor alpha chain IGF-I receptor beta chain) (Cox, et al., 2009). Insulin, IGF-I and hybrid receptors all exhibit ligand-dependent tyrosine kinase activity, and activate the AKT and MAPK signalling pathways. IGF-IRs can be activated by physiological concentrations of IGF-I and IGF-II, but not by insulin (Cox, et al., 2009). Classically, IRs binds only insulin, but now there is evidence that there are two insulin receptor isoforms (IR-A and IR-B), and that IR-A can bind IGF-II. While IGF-I can bind to hybrid receptor (IGF-IRs + Either isoforms of insulin receptor) (Gallagher & LeRoith, 2010). Insulin can bind with either of the isoforms IR-A and IR-B. Hybrid receptors bind IGF-I and IGF-II, and their affinity for insulin is an active research topic. Prostate cancer displays a mixture of IRs (insulin receptors) and hybrid receptors (IGF-IRs) on their surface (Bashan et al., 2009). Higher levels of the IGF-IR are expressed in the majority of primary and metastatic prostate cancer tumors. Further studies have suggested that IGF-IR signalling is required for survival and growth in androgen independent prostate cancer cells (Hellawell et al., 2002). When immortalized prostate cancer cells mimicking advanced disease are treated with the IGF-IR ligand, IGF-I, the cells become more motile. Greater expression of insulin receptor isoforms in human prostate cancers and increased levels of insulin receptors support the possibility that prostate cancer tissue can respond to increased levels of insulin (Jones et al., 2004).

2.3.2 Insulin signaling mechanism

Insulin generates free radicals by an NADPH, phosphatidylinositol 3-kinase dependent mechanism in human skin fibroblasts (Ceolotto et al., 2004). Insulin stimulation of target cells elicits a burst of H_2O_2 that enhances tyrosine phosphorylation of the insulin receptor and its cellular substrate proteins as well as distal signalling events in the insulin action (Loh et al., 2009). Human prostate cancer frequently shows both increased H_2O_2 and Nox1 and increased Nox1/H_2O_2 in an animal model system correlates with increased tumorigenicity (Jones, et al., 2004). High (millimolar) concentrations of H_2O_2 activate insulin signalling pathway and induce typical metabolic actions of insulin (Mahadev et al., 2004). Mice lacking

one of the key enzymes involved in the elimination of physiological ROS (Reactive Oxygen Species), glutathione peroxidase 1 (Gpx1), were protected from high-fat-diet-induced insulin resistance. The increased insulin sensitivity in Gpx1$^{-/-}$ mice was attributed to insulin-induced phosphatidylinositol-3-kinase/Akt signaling and glucose uptake in muscle and could be reversed by the antioxidant N-acetylcysteine (Giovannucci, 2005).

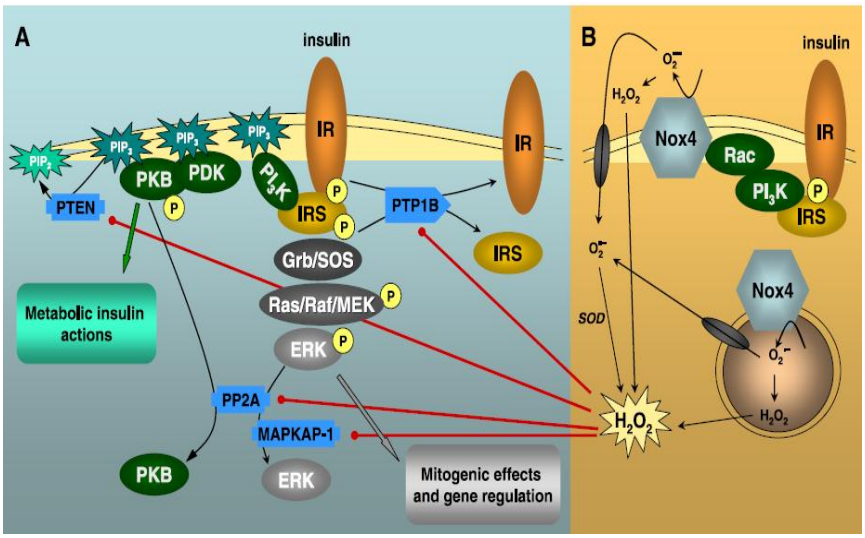

Figure 2.4: Insulin Signaling mediated by H_2O_2 (Bashan N et al 2009)

Main underlying cellular mechanism for the insulin mimetic effect of millimolar H_2O_2 concentrations is the inhibition of the catalytic activity of various protein and lipid phosphatases (Schmitt et al., 2005). H_2O_2 increase in adipocytes and muscle, increases glucose uptake and in adipocytes also results in GLUT4 translocation and lipid synthesis. Intracellular single-domain enzyme PTP1B is a member of an extensive family of protein tyrosine phosphatases (PTPs), is the major tyrosine phosphatase (Bashan, et al., 2009; Hayes & Lockwood, 1987). Lipid phosphatase PTEN is also an important regulator of insulin signalling because this catalyzes the dephosphorylation of 3-phosphoinositides, (lipid second messengers essential for insulin signal transduction). Reactive species such as H_2O_2 are ideal second messengers as they have short half life. Their function is also limited in time and space; they can initiate chain reactions, and can be generated and regulated through regulatory mechanisms (Mahadev et al., 2001). Recent epidemiological studies have shown connection between diabetes mellitus type 2 and increased cases of cancer. Excess body weight or fat is related

with increase in serum glucose, triglycerides and insulin level. These factors are correlated with the initiation and progression of cancer.

2.4.1 Oxidative Stress

Oxidative stress is caused by imbalance of reactive oxygen or nitrogen species production and cellular antioxidant system dysfunctioning. Reactive oxygen species are by product of normal cellular metabolism. But ROS level increases abruptly under stress conditions (Venkataraman et al., 2004). ROS generation is dependent upon various factors. H_2O_2 acts as an essential component of signaling pathways, but it's temporal and spatial formation in cells determine how it will act and affect various cellular processes. Cellular redox status regulate the activity of transcription factors and activators, hence gene expression depends on ROS level. For the normal cellular signalling ROS plays an essential role (Miao & St Clair, 2009). Redox potential alteration acts as a mutagen which enhances tumorigenesis. This oxidant introduces single and double strand breaks and base modifications in DNA (Iijima et al., 2009). ROS induced oxidative damage includes apurinic/apyrimidinic and oxidized purine and pyrimidine DNA sites (Kryston et al., 2011). Increased ROS can increase cell proliferation, induce genetic instability, somatic DNA mutations and can also promote angiogenesis in cancer cells (Khandrika et al., 2009). Protein folding can also be affected by oxidative stress. 8-OHdG (8-hydroxy -2'-deoxyguanosine) is the prime example of base modification in oxidative stress. Enzymatic and non enzymatic antioxidants maintain redox homeostasis at cellular level. Enzymatic antioxidant defence system includes glutathione peroxidase, catalase, and superoxide dismutase, while non enzymatic antioxidants includes represent flavonoids, α-tocopherol, ascorbic acid, carotenoids and glutathione. Balance between activity and level of antioxidants is required for normal cellular functioning. Superoxide dismutase includes a family of enzymes which protect cells from oxidative damage (Valko et al., 2007). Oxidative damage can be triggered by extracellular stimulants, ionizing radiations as well as by intrinsic factors (Mccord, 1993). Superoxide dismutase catalyzes dismutation of O_2^- to H_2O_2. Further catalase in the peroxisomes converts H_2O_2 into H_2O and O_2 (Lim et al., 2005).

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2H_2O + O2$$

2.4.2 SOD (Superoxide dismutase)

In humans three forms of superoxide dismutase are found: SOD1, SOD2 and SOD3, which are localized in cytoplasm, mitochondria and extracellular compartment respectively. SOD1 is found as dimer, while other two isoforms are tetramer. Prosthetic group in SOD2 is Mn while in SOD1 and SOD3 Cu and Zn is found to be associated at the reaction centre (Floriano-Sánchez et al., 2010). Human SOD1, SOD2, and SOD3 genes are localized chromosome 21q22, 6q.25.3 and chromosome 4 respectively. SOD1and SOD2 genes consist of five exons interrupted by four introns. Human SOD2 gene has 90% homology in the coding region and shows unique genetic organization of the gene (Miao & St Clair, 2009). Promoter of the SOD2 gene lacks TATA and CAAT box but contains GC-rich motifs. SOD3 gene consists of three exons and two introns. Mouse SOD3 cDNA shows 82% homology to that of rat and only 62% to the human SOD3 (Extracellular SOD) (Miao & St Clair, 2009). This is a well known fact that intracellular redox balance regulates cancer cell behaviour e.g. prostate cancer cell invasion. Recent studies have shown that not only intracellular redox state but extracellular redox state also plays a significant role in metastasis and progression of cancer. Extracellular space generally is found to be having more oxidized redox state as compared to interior of the cells (Chaiswing et al., 2007). Extracellular redox state is regulated by the interplay of various enzymes e.g. NADPH oxidase found at the plasma membrane, Extracellular superoxide dismutase (SOD3), Thioredoxin1, Extracellular glutathione peroxidase 3 etc (Chaiswing, et al., 2007). It has been found that over expression of extracellular glutathione peroxidase results in inhibition of prostate cancer cells invasion or metastasis. In malignant PC-3 cells MnSOD is found at lower level as compared to benign cancer cells. Thermotherapy or heat shock also generates ROS in prostate cancer cells and it culminates in cell death in two ways, either through apoptosis or necrosis. Inhibition of SOD3 activity in prostate cancer cells (PC-3) by treatment with Diethyldithiocarbamate (DDC) results in increase in superoxide level which induces cell death. Diethyldithiocarbamate chelate Cu metal ion hence inhibits the activity of extracellular superoxide dismutase (Moriyamaâ€• Gonda et al., 2002).

2.5 Matrix metalloproteinase (MMPs)

(MMPs) is secreted from cells into extracellular space, where it can degrade extracellular matrix. In the morphogenesis process MMP plays an important role. Matrix metalloproteinases (MMPs) play a major role in angiogenesis as well as in Vasculogenesis in cancer. Matrix metalloproteinases are family of Zn dependent endopeptidases, which can degrade various components of extracellular matrix.

2.5.1 MMP structure

It involves various domains as

i) Signal domain-It is required for the movement of peptide into the rough endoplasmic reticulum during synthesis.

ii) Propeptide domain-It maintains the latency in enzyme until it is disrupted.

iii) Catalytic domain-It has highly conserved Zn binding region which houses all the catalytic activities mediated by MMP.

iv) Hemopexin region-Substrate specificity is determined by this domain. Lastly, hinge region functions in mediating the transfer of substrate from hemopexin domain to catalytic domain.

Membrane type matrix metalloproteases posses an additional transmembrane domain and intracellular domain. MMPs are secreted in the inactivated or latent form and activated extracellularly by the proteolytic cleavage of propeptide. Membrane type MMP is activated by cleavage mediates by furin (Roy et al., 2009).

2.5.2 Role of MMPs in metastasis and angiogenesis

Depending upon the substrate, which MMPs can degrade, they have been categorized into different subtypes such as gelatinases, collagenases, stromelysins and matrilysins. Extracellular matrix components mainly include collagen and other proteins such as laminin, entactin and proteoglycans that form the basement membrane. Tumor cells over express or induce stromal cells to over express MMPs in order to degrade basement membrane and invade other tissues. Extracellular matrix degradation product, such as collagen IV and laminin, exhibit activities in cell signalling, which results in enhanced migratory activities in cancer cells. MMPs substrate includes not only cell adhesion molecules e.g. E cadherin,

integrin, but it also involves degradation of precursors of inhibitors of angiogenic factors and growth factor precursors. Epithelial to mesenchymal transition, a hall mark of metastasis stage of cancer, is also regulated by expression of MMPs. Activation of growth factors and degradation of cell adhesion molecules can be the main reasons or the mechanisms for epithelial to mesenchymal transition. Matrix metalloproteases promote angiogenesis by mediating the release of angiogenic factors stored in the extracellular matrix e.g. VEGF (vascular endothelial factor) Degradation of perlecan by MMP-2 and 9 results in the release of basic fibroblast growth factor from the extracellular matrix (Roy et al., 2009). Various MMPs degrade extracellular matrix and also mediate angiogenesis. Expression of MMP-2, 9, 15 and 26 have been positively correlated with prostate cancer. MMP-2 and 9 is positively expressed in plasma in metastatic prostate cancer. They can be used as a marker as level of MMPs 2 and 9 decreases significantly after therapy. In addition MMP-2 in urine can be used to differentiate between prostate and other type of cancer such as bladder cancer (Deryugina & Quigley, 2010). Synthetic inhibitors of MMPs reduce tumor growth as well as metastases in rat prostate cancer model. While low level of MMP-9 in prostate carcinoma reduces lung metastasis but not affect tumor growth. MMP-2 deficiency results in reduction of immature blood vessels hence reduced tumor burden because angiogenesis (neovascularization) is a critical step in the tumor growth (Littlepage et al., 2010). Overexpression of MMPs by transcriptional enhancement and transgene construct lead to tumor invasion in distant site in vivo model, while in-vitro it can be confirmed by matrigel or ECM invasion (Deryugina & Quigley, 2010). Cancer cell migration, invasion and cancer cell mediated tissue remodelling are mainly linked with MMPs. Primary as well as secondary metastases have been correlated with the elevated expression of various MMPs e.g. MMP-1,-2,-3,-7,-9,-13,-14 etc (Deryugina & Quigley, 2006). Antisense mediated degradation of MMP-9 in aggressive prostate cancer cells results in inhibition of the gene expression of angiogenic factors such as VEGF and ICAM. Treatment of gelatinase inhibitor (SB-3CT) in prostate carcinoma animal model significantly reduces intratumoral vascular density in bone metastasis. It highlights the role of gelatinases in angiogenesis (Deryugina & Quigley, 2006).

Studies pertaining to prostate cancer have shown that over expression of extracellular SOD3 reduces the expression of various forms of matrix metalloproteinases, hence metastasis or cancer cell invasion is also inhibited in vitro (Masur et al., 2010). It has also been reported in studies that Manganese superoxide dismutase over expression inhibits the growth of androgen independent prostate cancer cells PC-3 (Venkataraman et al., 2004). Growth of the tumours and metastasis involves formation of new vasculature. This process is of two types 1) Angiogenesis e.g. formation of new blood vessels from pre-existing vasculature. 2) Vasculogenesis e.g.formation of new blood vessel from endothelial progenitor cell. In the normal physiological process Angiogenesis takes place during estrogen cycle in female reproductive tract and also during wound healing (Deryugina & Quigley, 2010). In a benign solid tumor angiogenesis is an important step towards metatstatic stage in cancer.

2.6 Objectives of the study:

On the basis of the above literature revealing the role of insulin in increasing risk of cancer and complex role of reactive oxygen species in carcinogenesis our focus is to find out their relationship in prostate cancer. So the present study is planned with the following objectives:

- To check effect of insulin treatment on proliferation of PC-3 cells
- To monitor ROS level after insulin treatment
- To determine SOD gene expression level after insulin treatment

Chapter- III

Materials and Methods

3. Materials and Methods

This Chapter of dissertation includes description of material, protocols, chemical reagents and instruments used in the experimentation. All chemicals and reagents used during experimentation were of cell culture grade.

3.1 Cell Culture Treatments

Prostate cancer cell line PC-3 was obtained from National Center for Cell Sciences (NCCS, Pune, Maharastra, India). Cell lines were cultured in DMEM (HiMedia/Gibco, invitrogen) media supplemented with fetal bovine serum (FBS) (Gibco, invitrogen) and streptomycin and Penicillin antibiotics (HiMedia, India / Gibco, invitrogen).

Cells were cultured at 37^{o}C with 95% humidity and 5%CO_2. Cell lines were maintained and subcultured according to their doubling times and incubated in CO_2 incubator (Eppendroff, UK). For treatments 5000 cells per well were seeded in 96 well micro titer plate (Tarson) and 200000 cells in 6 well plate.

3.1.1 Treatments Preparations:

Recombinant human Insulin (Biocon, India) were prepared in sterile 1x PBS (HiMedia).

Table 3.1: Treatment Plans

S.No.	Cell line	Control	Treatments Groups	Concentration (nM)	Incubation period (hours)
1.	PC-3	No insulin	Insulin with FBS	10, 100, 1000	24, 48, 72
2.	PC-3	No insulin	Insulin without FBS	10, 100, 1000	24, 48, 72
3.	PC-3	No Insulin	Insulin with FBS	50,500,1000, 2000	48

3.2 MTT Assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution was prepared by dissolving in the PBS at 0.5mg/ml concentration and then 100ul per well was added to cell culture micro titer plate, incubated for 4hours at 37^{0}C. Formazan complex formed by active cells is solubilised with DMSO (200ul per

well) and absorbance measured at 570 nm (Aft et al., 2002) in Microplate reader (Biorad USA). Serum starvation was given for 12 hours. After treatment period MTT assay was done to check proliferation in PC-3 cells. .

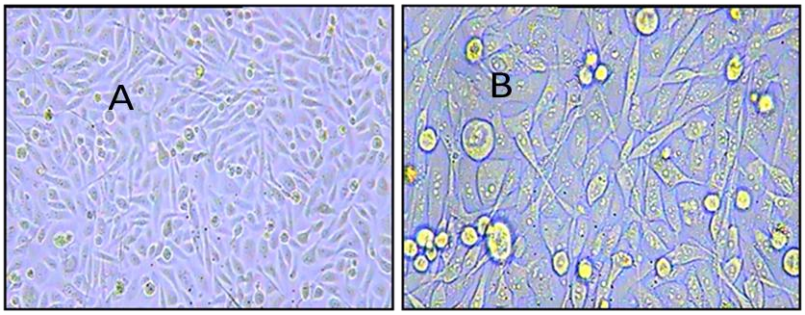

Figure 3.1: **PC-3 cell line. (A) (B**) Microscopic image of confluent PC-3 cell line in inverted compound microscope at 10X and 20X respectively.

3.3 ROS Assay

PC-3 cells were harvested from cell culture flask at 80% confluence by using trypsin. After cell counting in the neubauer chamber, cells were seeded in 24 well plate (100000 cells per well). Serum starvation of 12 hours was given and then four different insulin treatments 50nM, 500nM, 1000nM and 1000nM were given for 24 hours.

3.2.1.1 Fluorescence intensity measurement:

For fluorescent staining of the O_2^- and H_2O_2 (molecular probes) in PC-3 cells, DCFDA (Sigma) dye was used for 30 minutes. This was followed by fixing with 2% Para formaldehyde and then mounting on clean slides and observing under fluorescent microscope (FSX 100 Olympus). Image Pro software (Nikon) was used for fluorescent intensity measurement. For intensity measurement 10 cells from each picture were selected to measure mean intensity. Since each treatment had three independent pictures, so total mean intensity of 30 cells was averaged for calculation.

3.2.1.2 Spectrophotometric analysis:

For spectrophotometric analysis, equal numbers of cells were seeded. After the mentioned treatment periods, cells were suspended in solution using trypsin and washed with 1X PBS. Staining was done by incubating the cells with DCFDA dye

in 1X PBS on ice for 20 minutes. Cells were than washed three times with 1X cold PBS and OD was taken in UV-Visible Spectrophotometer (Shimadzu, Japan. Model: UV-2450). The measurement parameters for DCFDA were 488 nm excitation and 520 nm emission (Chen et al., 2003).

3.3. RT PCR

PC-3 cells were harvested from cell culture flask at 80% confluence by using trypsin. After counting in the neubauer chamber, cells were seeded in 6 well plate (200000 cells per well). Serum starvation of 12 hours was given and then four different insulin treatments 50nM, 500nM, 1000nM and 2000nM were given for 24 hours. PC-3 cells from 6 well plates were trypsinised and centrifuged to collect a pellet of cells. Cells were processed as per the manufacturer's instructional protocol for RNA isolation (Genei India). RNA was quantified using Nano Drop spectrophotometer to check quality and quantity of RNA. cDNA synthesis was performed by using cDNA synthesis kit (Genei, India) as shown in Table(3.2). The RT - PCR conditions were 42°C for 1hour cDNA synthesis followed by 95°C for 5 minute for mRNA denaturation.

Table 3.2: RT PCR components used for cDNA synthesis

RT 1X Reaction mixture	
Name of Components	**Quantity**
RNA	**5µl**
5X assay buffer	**4µl**
30mM dNTPs	**0.5µl**
Oligo dT	**0.5µl**
Random hexamer	**0.5 µl**
100M DTT	**1 µl**
RNase In	**0.5 µl**
AMV RT	**0.5 µl**
H_2O	**7.5 µl**
TOTAL	**20µl**

PCR amplification of SOD gene was performed by using PCR kit (Applied Biosystem, USA). PCR components used in the experiment are shown in Table (3.3). The following PCR conditions were used for SOD genes: 94°C for 30 sec (for enzyme activation and target denaturation), followed by 28 cycles of 94°C for 30 sec., 55°C for 30 sec. and 72°C for 30sec ; and a final extension at 72°C for 5 min.

Table 3.3: Components used in the PCR for SOD gene amplification

SOD 1X PCR reaction	
Name of the component	**Quantity used**
10X PCR Buffer	**1µl**
MgCl (25mM)	**1 µl**
dNTPs(2.5mM)	**1 µl**
Taq Polymerase	**0.3 µl**
Template	**1 µl**
Water	**1.7 µl**
Forward Primer	**2 µl**
Reverse Primer	**2 µl**
Total	**10 µl**

Primer used

SOD gene primers were designed from sequences available in NCBI by using GENE RUNNER software.

Table 3.4: Primer sequences of SOD gene isoforms 1, 2

Primer name	**Sequence (5' → 3')**	**Product Size**
SOD1 Forward	ATTCTGTGATCTCACTCTCAGG	215 base pairs
SOD1 Reverse	GCTAGCAGGATAACAGATGAGT	
SOD2 Forward	GTGACTTTGGTTCCTTTGAC	165 base pairs
SOD2 Reverse	GAATAAGGCCTGTTGTTCCT	

3.5 Gelatin Zymography

Zymography is SDS PAGE modified technique in which substrate is copolymerized with acrylamide gel to detect the activity of corresponding enzyme. In gelatin zymography gelatin is polymerized in acrylamide gel to detect the activity of MMPs. In this experiment MMPs level was checked in PC-3 cells after insulin treatment. PC-3 cells were harvested from cell culture flask at 80% confluence by using trypsin. After cell counting in the neubauer chamber, cells were seeded in 96 well plate (5000 cells per well). Serum starvation was given for 12 hours and followed by three different insulin treatments 10nM, 100nM and 1000nM for 24 hours. Media was collected from each well and concentrated in the vacuum concentrator (Eppendorf Germany).

10% polyacrylamide gel was prepared in 0.1% SDS containing 1mg/ml gelatin. Culture supernatants diluted with 6:1 7X sample buffer (17.4%SDS, 7% sucrose and phenol red in 1M tris-Cl pH6.8). Equal amount of protein was loaded in each well and subjected to electrophoresis at 200V, 50mA for 45 minutes at 4°C. Six washes were given in 2.5% TritonX100 in water (5 minute each).Three washing were given in PBS (10 min for each washing). Gel was placed in PBS pH7.4 containing 0.9mM $CaCl_2$, MgCl2 and 0.001mM $ZnSO_4$ and incubated overnight at room temperature on dancing shaker (Tarsons). Gel was stained with coomassie brilliant blue and then de-stained till clear bands were visible.

3.6 Data analysis

Data collected was statistically analysed using Sigma Plot version 11.0. Level of significance was evaluated using student's t-test and for multiple comparisons. One way ANOVA was performed with Tukey's test and level of significance considered at $P \leq 0.05$.

Chapter -IV

Results

4. Results

The present study investigates role of insulin in proliferation of prostate cancer cell line PC-3. The rationale behind the study is that there may be increase in proliferation due to increased metabolism reflected by hyper ROS production and higher SOD expression. Insulin treatment dose and time was selected after a wide range scan of concentration and time dependent effect on cell proliferation.

4.1 Insulin induces PC-3 cell proliferation

Since various growth factors which are present in cell culture grade serum can also affect the cellular proliferation of cultured cells. The first aim of the study was to investigate the effect of growth factors present in serum on insulin induced cell proliferation. In order to elucidate the exact role of serum, two sets of experiments were setup (1-with serum & 2-without serum) and cells were treated with different doses of insulin for 24, 48 and 72 hours. Cell proliferation was measured using % viability (transformed MTT absorbance). The results of insulin treatment in presence of serum revealed that PC-3 did not show any appreciable increase in the proliferation from initial 10nM to 100 nM concentrations, later it increased statistically (at 1000nM insulin concentration) up to 1.1 fold in 24 hrs. No change in proliferation was observed after 48 hrs with similar treatments. But 72 hrs treatments did change the rate of proliferation significantly (Fig 4.1 B).

On the other hand in serum free culture set up, proliferation of Insulin treated PC-3 cells showed significant cell proliferation (1.2 fold) at 10nM treatment over control. 48 hours treatment also significantly enhanced the cell proliferation 1.13 and 1.11 fold at 100nM and 1000nM insulin doses respectively. Further increasing the time up to 72 hrs did not affect the proliferation rate over control (Fig 4.1 A).

In conclusion it was observed that 24 hr insulin treatment causes proliferation at initial concentrations but later when the duration of increased to 48 and 72 hrs, the proliferation rate decreased substantially. It is quite evident that PC-3 is more responsive without serum for 24 hrs but further inhibition in cell growth may be due to lack of nutrients. Whereas insulin treatment with serum showed later effect on

cell proliferation with time, justifying the role of serum in time dependent dose.

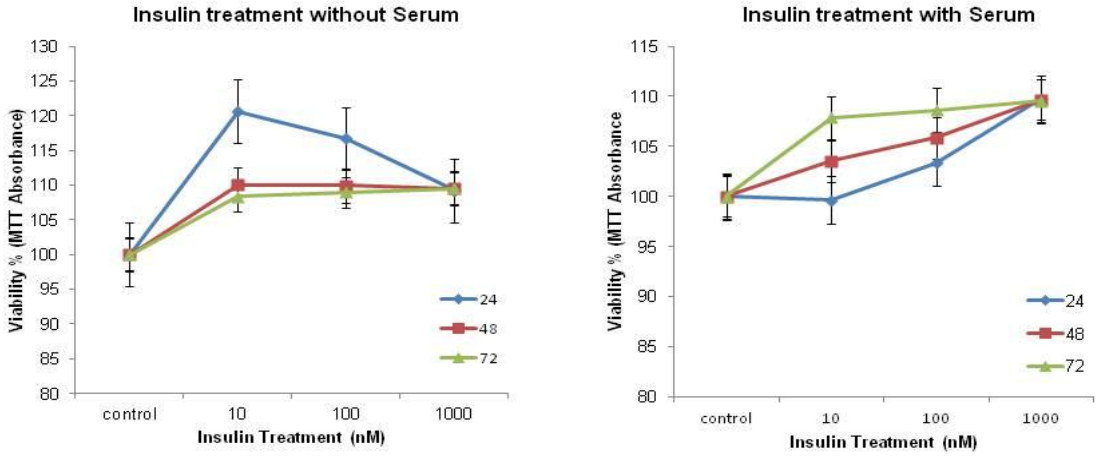

Figure 4.1: Comparative cell proliferation with and without FBS (A without & B with serum)

4.2 Dose dependent effect of insulin on PC-3 cell proliferation

A broad range of insulin dose was tested on PC 3 cells (with FBS) at 48 hrs time interval. Previous study has shown almost no difference in PC-3 cell proliferation between FBS and without FBS treatments of Insulin at different time periods. Broad range insulin treatment was found to be highly effective. At 10nM 1.5 fold increase in PC-3 proliferation was observed as compared to control. Increase in the insulin doses further enhanced cell proliferation and at 2000nM it increased up to 2.15 fold. It is quite evident from these observations that insulin is promoting PC-3 cells growth to a significant level.

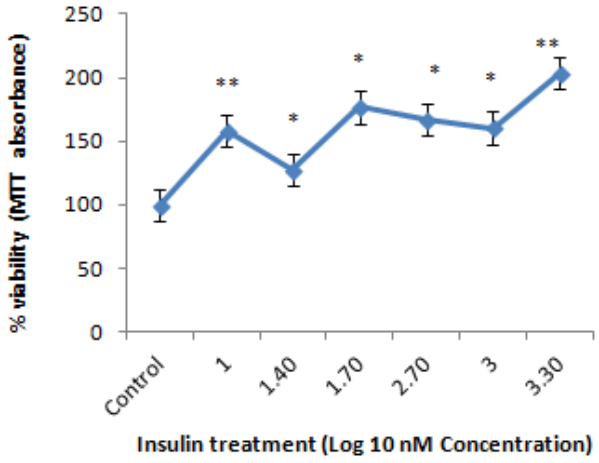

Figure: 4.2: PC-3 cell proliferation after 48 hours treatment of insulin (with FBS). Insulin doses used in the experiments were 10nM, 25nM, 50nM, 500nM, 1000nM and 2000nM. *, P≤0.05 **, P≤0.01

4.3 Insulin increases Free radical stress in PC 3 cells

Since insulin can directly affect cellular metabolism in number of ways which might be reflected upon increased cell proliferation. Free radicals are closely related to cell metabolism, whose alteration may change level of ROS inside a cell. Our hypothesis is that with increased cell metabolism, there will be hyper production of free radicals, which in-turn may lead to cell transformation (hyper-proliferation) through direct DNA damage or through dysregulation of various signalling pathways. We thus aim to investigate the status of free radicals in PC-3 cells with or without insulin treatment.

PC-3 cells were treated with different insulin doses for 24 hours and stained with DCFDA dye for detection of ROS by fluorescent microscope followed by comparison of cell intensity (10 cells per treatment in triplicate). Fluorescent images for free radical levels measured by DCFDA (in green; Fig. 4.3) show a dose dependent increase.

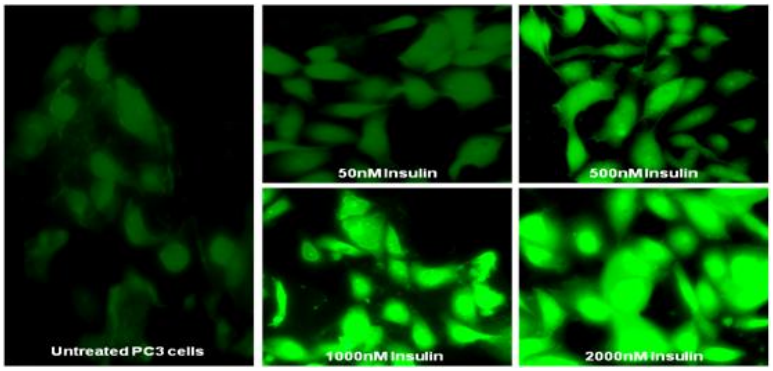

Figure 4.3: Fluorescent images of PC-3 cells treated with different insulin doses for 24 hours and stained with DCFDA dye for detection of ROS. Images were captured in Olympus fluorescent microscope (FSX 100) at 20X.

We next wanted to compare the relative staining intensity from all the samples. The staining was done in triplicate and from each sample, 10 cells were used to measure intensity using Image-Pro software from Nikon (10 X 3 = 30 cells per time point). The plotted graph showed progressive increase in ROS at higher insulin doses. There was 1.76, 2.18, 2.36 and 2.75 fold increase in ROS staining intensity (at 50nM, 500nm, 1000nM and 2000 nM respectively; Fig. 4.4) as compared to control PC-3 cells.

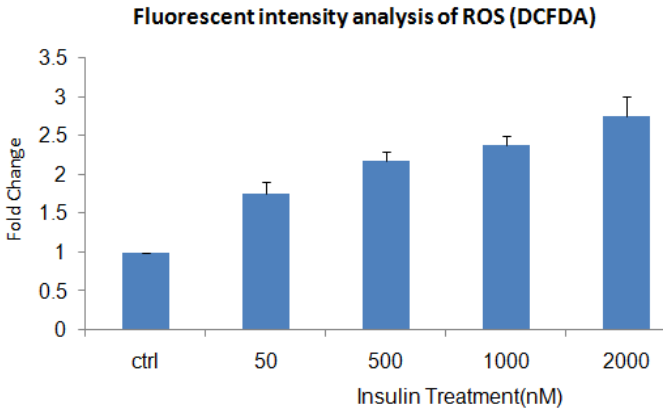

Figure: 4.4 Graphical representation of fluorescent intensity analysis of PC-3 cells for ROS detection after insulin treatment.

We further processed the DCFDA stained cells for spectrophotometric analysis. Our result showed that ROS level increases with the higher dose of insulin. Increase in ROS level was estimated as 2.22, 2.54, 2.39 and 2.68 fold in 50nM, 500nm, 1000nM and 2000nM insulin treated cells over control (Fig. 4.5). Statistically, this increase was highly significant at 50nm, 500nM, 1000nM and 2000nM as compared to control. Optical density was transformed to fold intensity, which was statistically significant over control and within treatments. Increase was significant at 50nm, 500nM and1000nM respectively.

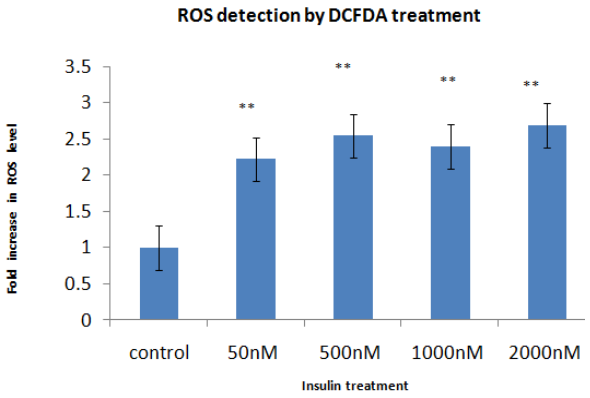

Figure 4.5: Spectrophotometric analysis of Insulin treated PC-3 cells stained with DCFDA for ROS detection. **, P≤.01

4.4 Superoxide dismutase expression with insulin treatment

Till now our results show that insulin treatment caused an increase in free radical levels. Hyper ROS can be harmful in number of ways like causing direct DNA damage, formation of protein aggregates as well as dysregulation of cell signalling pathways, so it's scavenging becomes of utmost importance. There are number of enzymes involved in scavenging like superoxide dismutase family. So our next goal was to investigate the effect of ROS increase on expression of SOD family members (SOD1 & 2).

Insulin treatment doses were selected on the basis of preliminary study of proliferation mediated at these concentrations. Insulin treatments of 50nM 500nM 1000nM and 2000nM were given to PC-3 cells for 24 hours time period to check the superoxide dismutase expression. RNA isolated from treated and untreated cells were subjected to two steps RT PCR (cDNA synthesis followed by PCR with SOD specific primers). The PCR product was viewed in 1.5 % agarose gel. Gel image was taken in geldoc (Biorad) and at the same time densitometric analysis was also carried out to check the band density. SOD1 PCR product was of 215bp (base pairs), while SOD2 PCR product was 165bp in length. In general SOD1 expression increased at 50nM insulin treatment but at higher concentrations it decreased appreciably (Fig 4.6 A). Further increase in insulin level may be causing oxidative imbalance. Densitometric analysis of SOD1 RT PCR revealed that at 50 nM and 2000 nM insulin treatment SOD1 expression increased 3.34 and 2.65 folds respectively as compared to control (Fig 4.6 B). Same trend was observed in SOD2 expression after insulin treatment. At 50nM insulin treatment SOD2 expression increased 1.07 fold, which later on decreased with higher insulin doses (Fig 4.6 C).

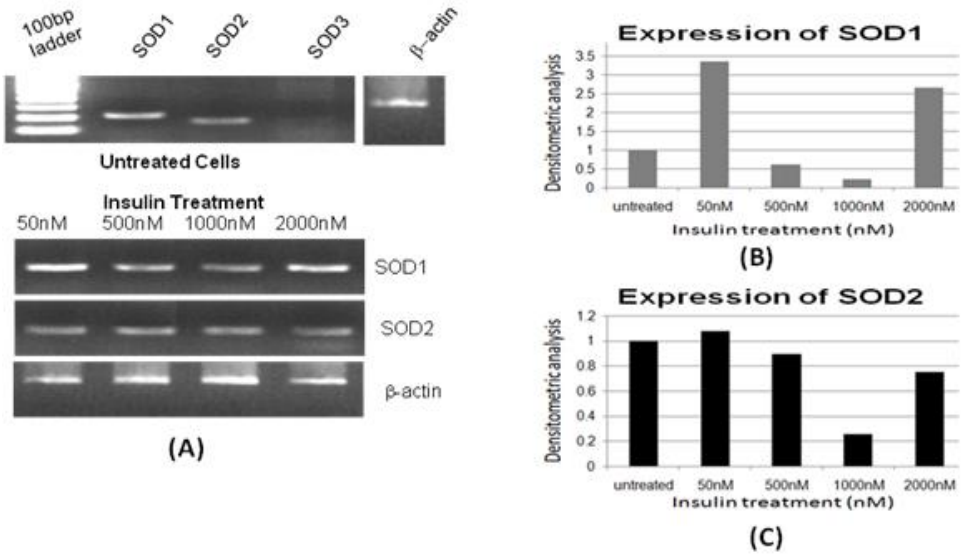

Figure: 4.6: **Superoxide dismutase expression in response to Insulin (A)** SOD 1, 2 and β actin, expression in the control (Untreated PC-3 cells) and treated cells.**(B**) Densitometric analysis of SOD1 expression **(C)** Densitometric analysis of SOD2 expression after insulin treatment at 50, 500, 1000 and 2000nM concentration after 24 hours.

4.5 MMP expression after insulin treatment

Since free radical can cause increased cell proliferation as well as dysregulated movement of the cells, we wanted to investigate whether insulin treatment can cause the same effect on PC-3 cells or not. MMPs are Proteases which degrade extracellular matrix and hence increase in the expression of MMPs can enhance metastasis in cancer. As extracellular matrix degradation is a major step in metastasis. Zymography was done to know whether insulin treatment has any effect on the expression level of MMPs. PC-3 cells were treated with 10, 100 and 1000nM doses of insulin and media was collected and processed for the zymography assay. Equal amount of protein was loaded in each well of gelatin zymography gel. Clear bands in the zymography gel shows MMPs has degraded their substrate (Gelatin), which is copolymerised with the gel. It is evident from this experiment that band clarity increased with higher doses of insulin (Fig. 4.7). So with the increase of insulin doses MMPs are secreted at higher levels and can have implications in metastasis.

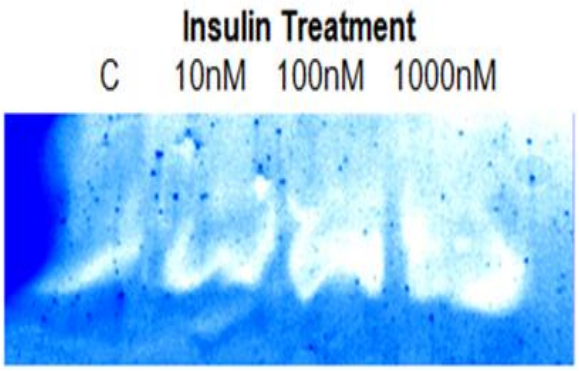

Figure 4.7: Zymography gel showing activities of MMPS at different concentrations of insulin in PC-3 cells

Chapter-V

Discussion

Several epidemiological studies have revealed association between Diabetes and cancer (Masur, et al., 2010). Exogenous infusion of insulin can have effect on proliferation of cancer cells and also promoting metastasis. Hyperglycaemia hyperinsulinemia and obesity are important factors that correlate diabetes with cancer. All these conditions influence cell proliferation. Approximately 8 to 18% people with malignancies also have diabetes (Sliwinska & Drzewoski, 2007). This is a well known fact that insulin acts as mitogen and increase cell proliferation. Present studies aims to investigate the role of insulin in prostate cancer cell proliferation, ROS production and Superoxide dismutase expression. Recent study showed that Insulin treatment increases PC-3 cells growth in a dose dependent manner. Also insulin antagonist S961 inhibits growth of PC-3 cells, which confirms that insulin acts as mitogen and has direct effect on proliferation (Vikram & Jena, 2012). Insulin analogues show mitogenic and antiapoptotic effect, although insulin analogues shows more pronounced effect on growth of various cell lines as compared to insulin (Weinstein, et al., 2009). Initially, the PC-3 cells were tested with and without serum and insulin treatment. Initial experiments depicts that insulin concentration at 10nM and 100 nM were not effective. PC-3 responded in same fashion with or without FBS. But without FBS cells fail to show any growth response with increase in the time. Cells grown with FBS were efficient in showing significant growth even in 72 hrs treatment. But when tested with broad range of insulin doses, PC-3 showed appreciable growth response even from 10 nM, which was significant over control. Since FBS contain essential growth factors that contribute to the survival of cells and hence can influence cell viability in 72 hrs treatment without FBS. Broad range of insulin dose was tested on PC-3 cells at 48 hrs time interval with FBS. A significant increase was recorded right from 10 nM (1.5 fold) to 2000nM (3 fold). It is quite evident from these observations that insulin is promoting PC-3 cells growth to a significant level. Oxidative stress results due to imbalance between steady state levels of intracellular pro-oxidants and antioxidants (I. M. Ahmad et al., 2005). Increased level of reactive oxygen species and metabolic defects is recognised as the most common feature in different cancers. Superoxide anion is the primary oxygen radical that is generated in the different biological processes. It is further metabolized into hydrogen peroxide (H_2O_2) and hydroxyl radical (HO$\cdot$) by plethora of antioxidant system at cellular

level. Hydroxyl radicals the most reactive form of ROS that causes oxidative damage to DNA and proteins in the cell. Low level of ROS promotes cell proliferation by activation of growth promoting signalling pathways in normal cells. Chronic oxidative stress plays a vital role in prostate cancer initiation and progression. Abnormally high level of ROS in cells leads to a state of oxidative stress that is linked with carcinogenesis and cancer progression (Chan et al., 2011). Increased lipid peroxidation and decreased efficiency of antioxidant system leads to production of epoxides. These moieties can covalently combine to DNA, RNA and protein which results in mutagenicity, cytotoxicity and carcinogenicity (MatÉs et al., 1999). This study was carried out to know the effect of higher insulin doses on the level of ROS. PC-3 cells were treated with different insulin doses up to 24 hours. Intensity calculation shows that ROS production increased at higher insulin doses. In terms of fluorescent intensity of PC-3 cells 1.76, 2.18, 2.36 and 2.75 fold increase was observed which confirms increase in ROS level. Spectrophotometric analysis of Insulin treated and DCFDA stained PC-3 cells showed 2.22, 2.54, 2.39 and 2.68 fold increase in ROS levels with increasing insulin concentration (50-2000nM). Previous studies pertaining to oxidative stress has revealed that cancer cells show higher ROS level as compared to normal cells. ROS generation in these cancer cells are related with NAD(P)H oxidase (Nox) systems and with mitochondria. Cross talk between ROS generation and tumorigenic potential shows that ROS can modulate tumor growth. Oxidative stress contributes to increase in aggressive and invasive behaviour in PC-3 cells (B. Kumar et al., 2008). So insulin not only increases proliferation but may contribute in aggressive prostate cancer due to increase in ROS level. DNA damage and modification in protein folding process caused by oxidative stress is other phenomenon which is related with carcinogenesis. Superoxide dismutase is an antioxidant enzyme which clears ROS from cells. Superoxide generated in intracellular processes is dismutated into hydrogen peroxide. SOD plays an important role in saving aerobic life, as ROS is continuously generated in the biological processes. But ROS level is kept low by cooperative activity of antioxidant system (Wiryana, 2009). SOD over expression leads to inhibition of tumor growth and decreased tumor aggressive nature (Plymate et al., 2003). RNA was isolated from treated and untreated cells and after cDNA synthesis PCR was

carried out with SOD specific primers. Densitometric analysis of SOD1 reveals that insulin is promoting SOD1 expression (3.34 folds) at initial dose of 50 nM. But with further increase in dose up to 2000 nM, decrease in the expression was observed. Further increase in insulin level may be causing oxidative imbalance. Densitometric analysis of SOD2 has also shown same trend increase in expression. At 50nM insulin treatment 1.07 fold increases was observed in comparison to control. Interestingly, further increase in treatment decreased the SOD2 expression level even lower than the control. Spectrophotometric analysis and fluorescent imaging revealed that ROS level increases with higher insulin doses, this increase in ROS level can be attributed to lower level of SOD gene expression. Free radicals can't be removed efficiently due to lower expression of SOD gene and it may lead to oxidative stress. So insulin may increase tumor's aggressive and invasive nature by modulating SOD expression and hence ROS level in cancer cells. Other studies regarding SOD expression in cancer cells have shown that different isoforms of SOD are expressed at varying levels depending upon the stage of cancer. Therapies involving modulation of SOD expression should be based on type and stage of cancer (Skrzycki et al., 2009). MMPs are family of proteases secreted by cells or associated with cell membrane. MMPs degrade extracellular matrix not only in the normal conditions but also has a role in the pathological conditions such as metastasis in cancer. Extracellular matrix (ECM) provides a structural framework to support cells and also mediates cell-cell and ECM-cell interaction. ECM is composed of proteoglycans, elastin and collagen. MMPs are synthesised in the latent form and converted later on in to active form (Ii et al., 2006). They can control microenvironment around cell through extracellular proteolysis. MMPs are divided into eight groups of which five are secreted while three are membrane associated (Hojilla et al., 2003). Our result shows that higher insulin doses can increase MMP_S expression. MMPs expression increased with increasing dose of insulin. Previous studies have shown that hyperglycaemia and higher insulin doses can increase migratory activity in various cell lines (Masur, et al., 2010). On the basis of these results we can say that insulin affects tumor growth and invasion by increasing MMP level and ROS level in cancer. All these factors can work in a synergistic way.

Chapter-VI

Summary

Hyperinsulinemia is correlated with increase in risk of cancer. Our study supports this fact and reveals that insulin acts as a mitogen and increases PC-3 cell proliferation. Increase in metabolism is reflected by the increase in ROS level, since ROS and SOD expression is modulated by insulin. Finally insulin also leads to increase in MMPs activity, which is related to metastasis. Taken together all these factors we can say that insulin supports tumor progression. In addition to this further studies are required to explore the role of SOD3 in ROS. Temporal and spatial localization of ROS determines its role, as ROS are also essential in signalling process but abnormal increase in ROS level can also have detrimental effect on cells. This complex relationship requires extensive studies.

Chapter-VII

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