TO STUDY THE EFFECTS OF INSULIN AND METFORMIN ON PC-3 CELL LINE

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By

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CERTIFICATE

I declare that the dissertation entitled "**To study the effects of Insulin and Metformin on PC-3 cell line**" has been prepared by me under the supervision of Prof. P. Ramarao, Administrative Guide, Dean, Academic Affairs, Central University of Punjab, Bathinda and Dr. Felix Bast, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

We certify that Mr. Pushpendra Singh has prepared his the dissertation entitled ”**To study the effects of Insulin and Metformin on PC-3 cell line**”, for the award of M.Phil. degree of the Central University of Punjab, under our guidance. He has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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ABSTRACT

TO STUDY THE EFFECTS OF INSULIN AND METFORMIN ON PC-3 CELL LINE

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Key words: Insulin, Prostate Cancer, Metformin and Prostate Cancer (PC-3) cell line

Dietary habits, genetic factors, hormonal factors and environmental factors are the independent risk factors for prostate cancer as well as diabetes. Androgen is the primary growth factor for the prostate cancer initiation and progression, however, non androgen peptide growth factor like insulin and insulin growth factor also involved in the prostate cancer as well as diabetes. Insulin and insulin growth factor are peptide that regulates metabolism, growth, cellular proliferation and apoptosis. The anti-diabetic drug metformin is rapidly emerging as a potential anti-cancer agent that improves insulin homeostasis and decreased growth and cellular proliferation of the prostate cancer cell line. Thus it is necessary to understand the growth promoting role of insulin on prostate cancer cell line and the possible influences of metformin on the proliferation of prostate cancer cell line in the presence and absence of insulin has been studied.

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Pushpendra Singh

List of Contents

List of Tables

List of Figures

List of Abbreviations

Sr. No.	Full form	Abbreviation
1	Prostate Cancer	PCa
2	Diabetes Mellitus Type2	T2D
3	Diabetes Mellitus	DM
4	Adenosine Monophosphate-Activated Protein Kinase	AMPK
5	Mammalian Target of the Rapamycin	mTOR
6	Insulin Receptor	IR
7	Insulin Receptor A	IR-A
9	Ethylene Diamine Tetra Acetic Acid	EDTA
10	National Centre for Cell Science	NCCS
11	Fetal Bovine Serum	FBS
12	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide	MTT
13	NanoMolar,	nM
14	MicroMolar	µM
15	Insulin Growth Factor	IGF
16	Insulin Growth Factor I	IGFI
17	Insulin Growth Factor II	IGFII
18	Insulin Growth Factor Receptor I	IGFIR
19	Insulin Growth Factor Receptor II	IGFIIR
20	Insulin Receptor Substrate Proteins	IRS
21	Phosphatidyl Inositol 3-Kinase	PIK3
22	PCa Cell Line-3	PC-3
23	Prostate Specific Antigen	PSA
24	Nutrient Mixture F-12	Ham's F-12

CHAPTER – I

1. INTRODUCTION

The incidence of cancer has emerged as a global problem on past decades due to increasing remarkable incidence of cancer in the world population. Cancer being the second most common cause of death is exceeded only by cardiovascular disease. Global Cancer Statistics, 2011 reported that about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide. Prostate, colorectal, female breast and lung cancer rates are 2 to 5 times higher in developed countries as compared to developing countries. It is also reported that breast cancer in females and lung cancer in males are the most frequently diagnosed cancers and the leading cause of cancer death for each sex in both economically developed and developing countries, except lung cancer is preceded by Prostate cancer (PCa) as the most frequent cancer among males in economically developed countries. PCa is the second most frequently diagnosed visceral cancer and the sixth leading cause of cancer death in males (Jemal et al. 2011).

The metabolic syndrome associated cancer is a severe health problem world-wide. The metabolic syndrome includes obesity, hypertension, dyslipidemia and hyperglycemia and is linked to insulin resistance and hyperinsulinemia. These factors are hallmarks of diabetes and cancer leading to increase diabetes associated cancer risk. Diabetes increases the risk of cancer initiation and progression but in the case of PCa its relation is inverse. Epidemiologic research suggests that diabetes mellitus (DM) is associated with reduced PCa risk. Several studies reported that diabetes decreases the risk of PCa (Darbinian et al. 2008, Velicer et al. 2006, Pierce et al. 2008). However, numerous epidemiological studies show a positive relationship between on metabolic syndrome and prostate cancer. Metabolic syndrome increases the risk of PCa development and progression of malignancies. Kasper and Giovannucci (2006), Reported a Meta analysis, which show that metabolic syndrome is associated with PCa malignancies. Genome wide association studies have also provided further support for a link between Diabetes Mellitus Type2 (T2D) and PCa risk, as variants in the HNF1B and JAZF1 genes have been shown to influence both

PCa and T2D risk, confirming the existence of shared genetic factors and suggesting related disease mechanisms (Frayling et al. 2008).

Although the detailed mechanism involve in the diabetic PCa remain largely unknown, Hyperinsulinemia in the presence of insulin resistance affects the carcinogenesis of PCa by direct or indirect manner. Indirect carcinogenesis via the IGF axis hyperinsulinemia, oxidative stress, inflammatory cytokine and sex hormones may contribute the development and progression of PCa.

Metformin is an antidiabetic drug but it also reduces development and progression of the cancer. The mechanism of action of Metformin involved down regulation of the insulin and insulin-like growth factor axis that means insulin and Insulin Growth Factor (IGF) involved in the development and progression of PCa. Antitumor activity of metformin is Adenosine Monophosphate-Activated Protein Kinase (AMPK) dependent as well as AMPK independent (Anisimov 2010). High concentration of insulin develops insulin resistance and increases the risk of PCa initiation and progression. The risk of cancer- related mortality is increased in those with high insulin levels or insulin resistance and cancers of the Prostate (Amling et al. 2004). Further it is also reported that Insulin may exert a mitogenic effect through Insulin-like Growth Factor-1 (IGF-1) receptors (Pollak et al. 2004). Insulin affects the metabolism and also produces the mitogenic response. There is a well established link between metabolic syndrome and PCa. Emerging research is characterizing this relationship further and delineating the specific role of insulin in promoting PCa tumorigenesis.

CHAPTER – II

2. REVIEW OF LITERATURE

2.1. Physiological roles of insulin and insulin-Like Growth Factor

Insulin and Insulin-like growth factor are peptide these regulate cellular growth, proliferation, metabolism, survival, glucose homeostasis, cell differentiation and apoptosis (Dupont and Holzenberger 2003). Insulin is a crucial regulator of metabolic pathways, glucose, carbohydrate, and fat homeostasis. Insulin is secreted by beta cells of pancreas during the rising blood glucose levels. When released by the beta-cells of the pancreas, insulin binds to tyrosine kinase receptors on the surface of Hepatocytes, adipocytes, and muscle cells, these are classic insulin responsive cells and express high levels of insulin receptors (Xu et al. 1998). Apart from that insulin receptor also express in the tissue like prostate gland tissue. Insulin is primarily involved in regulating metabolism but it also effects the normal growth of the cells. Insulin has metabolic as well as mitogenic potential. Metabolic potential of insulin maintain metabolic activity and glucose homeostasis, but the mitogenic potential increases the tissue specific cell proliferation, survival and metastasis of the disease (Sachdev and Yee 2007). Mitogenic effect of insulin on the prostate gland increases the risk of PCa. On the other hand, IGF it is growth hormones, its signaling plays a fundamental role in regulating embryonic growth and regulates specific differentiation in most adult tissues (Duan and Xu 2005). Expression of IGF 1 receptor is a tissue specific. IGF1 express in the prostate gland and its increase the growth of the cell, as well as cell proliferation and increase the risk of PCa initiation and progression. The insulin and IGFI receptors, are separate gene products, are structurally very similar.

2.2. Insulin and IGF ligands

Insulin and insulin growth factor have 40-80% homology so it is difficult to explain insulin and IGF1 ligand receptor interaction. Insulin/IGF signaling system is comprised of three ligands, IGF-I, IGF-II, and insulin itself and another hand these ligands interact with at least seven receptors: the type I IGF receptor (IGF-IR), the type II IGF receptor (IGFIIR), the insulin receptor A (IR-A), the insulin

receptor B (IR-B), hybrid receptors of IGF and IR-A, hybrid receptors of IGF and IR-B, hybrid receptors of IR-A and IR-B. Insulin and insulin growth factor ligands receptor interaction is briefly illustrated in Figure 1. Insulin in blood circulation called insulin ligand is a monomer consisting of two chains, an A chain of 21 amino acids and a B chain of 30 amino acids linked by two disulfide bridges (De Meyts and Whittaker 2002) . On the other hand, IGFs are small, single-chain polypeptide ligands (7-8 kD) that are derived from prepropeptides in a similar way to insulin, but contain the C-peptide bridge between B and A chains that is normally cleaved in insulin (Beauchamp et al. 2010).The mature IGF-I and IGFII peptides consist of B and A domains that are homologous to B and A chain of insulin (LeRoith and Roberts 2003).

2.3. Insulin and IGF receptors and signaling

Insulin action is mediated through its tyrosine receptor. The insulin receptor is a heterotetrameric protein consisting of two extracellular α-subunits and two transmembrane β-subunits (Dupont and LeRoith 2001). The binding of ligand to α-subunits of insulin receptor stimulates the intrinsic tyrosine kinase activity of the β-subunits of the receptor. This receptor has the ability to autophosphorylate and phosphorylate intracellular substrates is essential for the mediation of the complex cellular responses to insulin and activation of cascade of intermediate protein , The activated IR tyrosine kinase phosphorylates several substrates including insulin receptor substrate proteins (IRS1-4), Phosphatidyl Inositol 3-Kinase (PIK3), Akt, mTOR, MAPK and signal regulatory protein Family (Frasca et al. 2008). Insulin and insulin growth factor signaling is briefly illustrated in Figure 1.

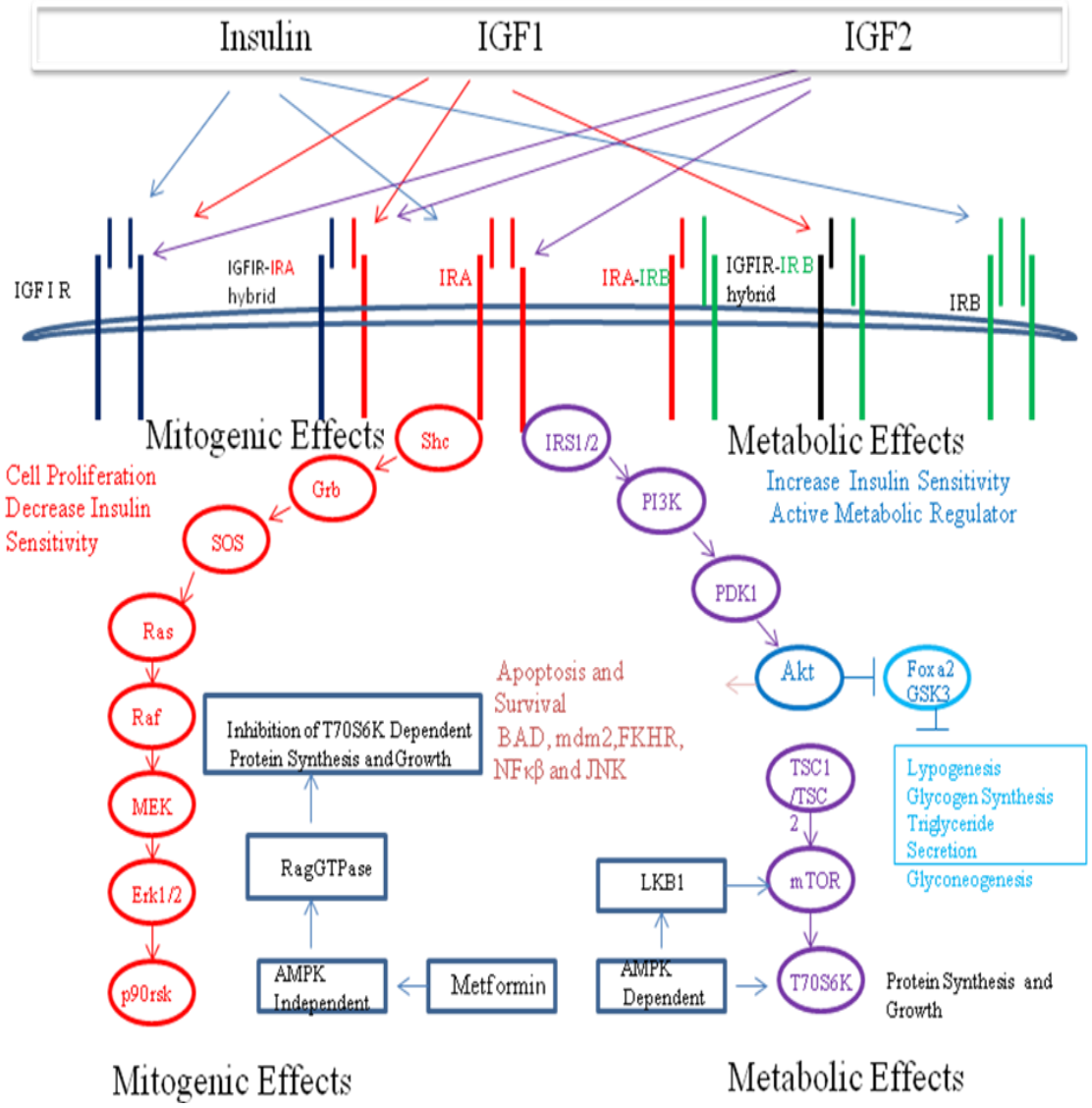


Fig.1:- IGF Axis –IGF axis is comprised of three ligands, IGF-I, IGF-II, and insulin itself and another hand these ligands interact with at least seven receptors: the type I IGF receptor (IGF-IR), the type II IGF receptor (IGFIIR), the insulin receptor A (IR-A), the insulin receptor B (IR-B), hybrid receptors of IGF and IR-A, hybrid receptors of IGF and IR-B, hybrid receptors of IR-A and IR-B. Structurally, all IR and the IGFR have two extracellular α-subunits and two transmembrane β-subunits that are jointed to each other by disulfide bonds. Insulin binds with high affinity to IR-A, IR-B and IGF-1R, IGF-1 binds to the IGF-1R and to the hybrid receptor IGF-1R/IR-A or IGF-1R/IR-B. IGF-2 binds to IR-A, IGF-1R or to IGF-1R/IR-A hybrid receptor. Insulin and insulin growth factor ligand binds to IGF1R, IR-A, and hybrid receptors of IGF and IR-A, mediates the mitogenic signaling pathway, while ligand binding to insulin receptor-B activates metabolic signaling. Binding to the hybrid receptors, leading to mitogenic or metabolic signaling, is determined by the IR isoform that formed the hybrid receptors.

2.4. Insulin and IGF in Human diabetic PCa

Insulin receptor (IR) expression is tissue specific, insulin responsive tissue such as liver; muscles have the high expression of insulin receptor. On the other hand non insulin responsive tissue such as prostate gland, have the high expression of insulin receptor but relatively little is known about the expression of insulin responsive and insulin unresponsive insulin receptor pathophysiology.

2.5. Insulin/IGF receptor expression on PCa cell lines

Insulin growth factor receptor 1 and Insulin growth factor receptor 2 both are highly expressed on the PCa cell line but expression of insulin receptor on PCa cell line is still awaited. Insulin and IGF receptor expression on PCa cell line PC-3 briefly illustrated in table1.

2.6. Insulin/IGF work on PCa cell lines

PCa need a cell line which mimics resistance to androgen therapy for study of the molecular mechanisms involved in metabolic syndrome and PCa. Numerous invitro studies have used PCa cell line as a model to assess the relationship to PCa and metabolic syndrome. There are three established cell lines, viz., PC3, DU145 and LNCaP that are routinely used in PCa research called classical cell line, However non classical PCa cell line viz., 1013L, 22Rv1, ALVA-55, ALVA-101, ARCaP, CWR-R1, DuCaP, DuPro-1, LAPC-4, MDA PCa 1, MDA PCa 2a, MDA PCa 2b, NCI-H660, PC-346C, PC-93, PSK-1, UM-SCP-1, and VcaP also used in PCa research. Here we are focus only insulin and insulin growth factor work on PC-3 cell line. Insulin/IGF work on PCa cell line PC-3 briefly illustrated in table 2.

Table 1:- Insulin/IGFs receptor expression on PCa cell lines.

Cell lines	IR-A, IR-B	IGF1R	IGF2R
PC3	**Yes** (Armakolas et al. 2010)	**Yes** (Kawabata et al. 2011)	**Yes** (Kawabata et al. 2011)
DU 145	**NA**	**High Expression** (Kawabata et al. 2011)	**Yes** (Kawabata et al. 2011)
LNCaP-FGC	**NA**	**Yes** (Bidosee et al. 2011)	**Yes** (Bidosee et al. 2011)
LNCaP-LN-3	**NA**	**Yes** (Letsch et al. 2003; Krueckl et al. 2004)	**Yes** (Letsch et al. 2003)
LNCaP-C4	**NA**	**Yes** (Krueckl et al. 2004)	**Yes** (Letsch et al. 2003)
MDA PCa 2a	**NA**	**Yes** (Goya et al. 2004)	**Yes** (Goya et al. 2004)
MDA PCa 2b	**NA**	**Yes** (Letsch et al. 2003)	**Yes** (Letsch et al. 2003; Goya et al. 2004)
22Rv1	**NA**	**Yes** (Kawabata et al. 2011)	**Yes** (Shukla et al. 2005; Cobb et al. 2009)

(Yes = Positive expression, NA = Negative expression)

Table 2:- Insulin/IGFs work on PCa

Work	References
Prostate gland has three zone peripheral zone, transition zone And stroma inner most. PCa occurs predominantly in the peripheral zone.	(Jiang et al. 2011)
Insulin-like growth factor-binding protein plays an important role in the insulin growth factor signaling. There are six Insulin-like growth factor-binding proteins. Among them some increase the cell proliferation and some do not. Insulin-like growth factor-binding protein-2 promotes PCa cell growth via IGF-dependent or -independent mechanisms.	(Uzoh et al. 2011)
Expression of androgen receptor high in androgen dependant cell line (LNCaP) and IGF1R and IGF2R expression levels high level in PC3 cells.	(Kawabata et al. 2011)
Androgen receptor expression determines the role of IGF-I and IGF-II. IGF-I was stimulated in the androgen-dependent LNCaP cells and IGF-II was stimulated in androgen-insensitive PC3 cells.	(Bidosee et al. 2011)
There are two pathways for the PCa initiation and progression. Suggests co targeting both pathways (**PIK3 and MAPK**) for PCa therapeutic interventions.	(Goc et al. 2011)

2.7. Antitumor action of Metformin

Metformin (1, 1-dimethylbiguanide hydrochloride) Begunide is an oral hypoglycemic drug. It is widely used in the treatment of type 2 diabetes and it is frequently referred to as an insulin sensitizer. It is increasingly being used to treat other conditions associated with insulin resistance, hyperinsulinemia and hyperglycemia. Metformin reduces the circulating insulin levels. (Gunton et al. 2003) reported that the mechanism of action of Metformin involves enhancement of signaling through the insulin receptor, which ultimately leads to improvement of insulin resistance, hyperinsulinemia and hyperglycemia. Metformin action on the insulin receptor leads to inhibitor of cell proliferation and survival of the cells (Holland et al. 2004). The pioneering work of Anisimov (2010) suggested that the mechanism of action of Metformin involved down regulation of the insulin/insulin-like growth factor axis, this may represent a novel paradigm for the treatment of human malignancies that reduces not only the initial cost of treatment, but the cost of treatment related complications that place such a heavy burden on health systems around the world.

The anticancer effects of Metformin are associated with both indirect (insulin dependent) and direct (insulin- independent) actions of the drug (Figure 2). The indirect, insulin-dependent effects of Metformin are mediated by the Adenosine Monophosphate-Activated Protein Kinase (AMPK) pathway which inhibit the transcription of key gluconeogenesis genes in the liver and stimulate glucose uptake in muscle, thus reducing blood glucose and insulin (Cusi et al. 1996) . Since insulin has mitogenic activity it affects cancer cells which have high level of insulin receptor expression indicating a potential sensitivity to the growth promoting effects of the hormone. Insulin-lowering effects of Metformin as a potential mechanism of action in the treatment of cancer. The direct, insulin-independent effects of metformin originate from LKB1-mediated activation of AMPK and a reduction in Mammalian Target of the Rapamycin (mTor) signaling and protein synthesis in cancer cells (Dowling et al. 2007).

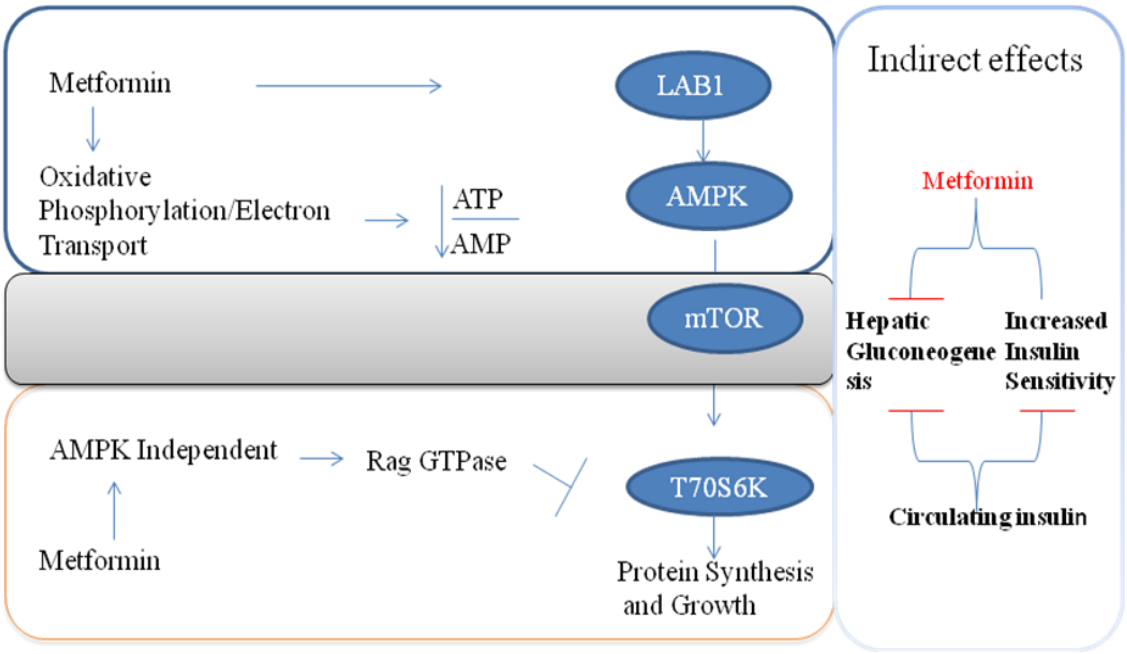


Fig.2:- Antitumor Mechanisms of metformin Action**.** metformin may activate AMPK via two separate mechanisms, the inhibition of oxidative phosphorylation/electron transport and subsequent decrease in the ATP/AMP ratio and/or the direct activation of LKB1. AMPK activation leads to blocking mTOR cell signaling that ultimately leads to inhibition of cell proliferation and survival. metformin may also work on AMPK independent; Rag GTPase mediated action of metformin resultant of this Inhibition of T70S6K Dependent cell proliferation, survival, Protein Synthesis and Growth of the cells. Apart from that metformin indirectly reduces the insulin level.

2.8. IR expression in physiological conditions

Major insulin target tissues, liver, adipose tissue, and skeletal muscle, are called insulin responsive tissue, but IR have also been found in the insulin unresponsive tissue like brain, heart, kidney, pulmonary alveoli, pancreatic acini, placenta vascular endothelium, monocytes, granulocytes, erythrocytes, and fibroblasts (Pandini et al. 2002). That means IR expression is not restricted to insulin target tissues. This suggests that insulin receptor may be functionally playing an important role in the non metabolic effects in addition to metabolic effects. Metabolic and nonmetabolic effects of insulin receptor are supported by the effects of insulin on growth and development (Belfiore et al. 2009). Lifestyle, nutrition and exercise have been reported to influence insulin receptor expression (Mamula et al. 1990)**.** Co-expression of insulin receptor and IGF-I receptor in prostate gland increase the risk of PCa initiation and progression. Again insulin

receptors have two isoform, IR-A and IR-B, one is metabolic and other is mitogenic. (Cox et al. 2009), reported that presence of both IR-A and IR-B insulin receptor isoforms on primary human PCas. This finding discloses hypothesis that candidate treatment target for the PCa may be IGF-I and insulin receptors both.

2.9 Aberrant IR-A expression in cancer

Aberrant IR-A expression may contribute to the deregulated response of cancer cells by the insulin and IGFs in many ways -

a) IR over expression increases the sensitivity of insulin and increases the pleiotropic effects of circulating insulin, especially during hyperinsulinemic and insulin resistance.
b) IR-A over expression may be binds to insulin growth factor receptor 1 and IR-B and forming hybrids receptor. Hybrid containing IR-A (HR-A) may bind insulin, although with relatively low affinity (Pandini et al. 2002).

Recent epidemiological studies have demonstrated that both T2DM and obesity are associated with an increased risk for many forms of cancer, including cancer of the breast, colon, liver, pancreas, kidney, and others, (Fisher 2001; Strickler et al. 2001; Vigneri et al. 2009). These studies, together with the finding that IR-A is often aberrantly expressed in cancer cells, have strengthened the hypothesis that insulin resistance and compensatory hyperinsulinemia are a major link between diabetes and cancer (Pisani 2008)

CHAPTER – III

3. HYPOTHESIS, OBJECTIVES AND RATIONALE

3.1 Hypothesis

An elevated level of insulin or serum IGF-1 or testosterone predicts PCa risk. This hypothesis is deduced from the following understandings, as illustrated in Fig. 3.

1) Within prostatic tissue, an increase in free insulin concentration (prostatic hyperinsulinemia) leads to insulin resistance. With insulin and insulin growth factor receptors expressed in the prostate gland, high level of insulin bind to these receptors and initiates the mitogenic growth that ultimately lead to the development of PCa.
2) Prostatic hyperinsulinemia also increases the aromatase activity (Tsugaya et al. 1996). Aromatase activity has been reported to increase the free testosterone levels. Increased testosterone levels are in turn reported to cause initiation of PCa.

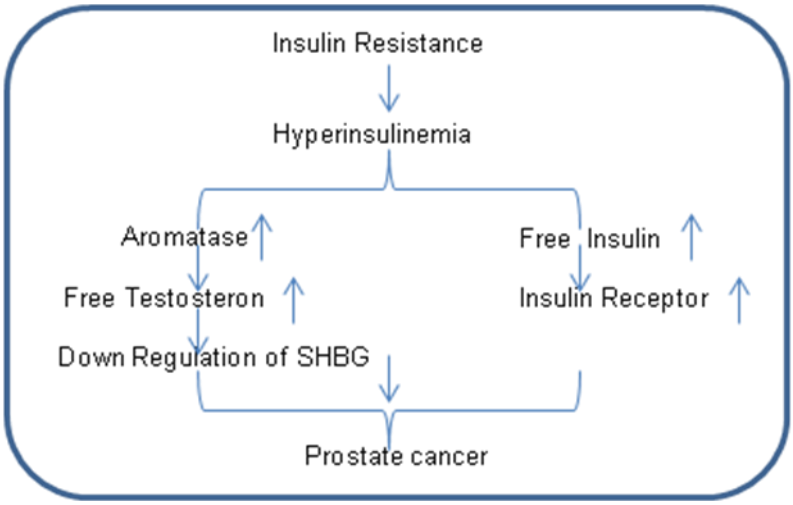


Fig.3:- Hypothesis. High level of insulin binds to IGF axis receptors and initiates the mitogenic growth that ultimately leads to the development of PCa.

3) Metformin is antidiabetic drug which reduces the free insulin level and it reduces the cell proliferation, survival of the cells. The mechanism involved in

the action of Metformin is down regulation of insulin and insulin growth factor by AMPK dependent and AMPK independent manner. Our hypothesis will be that metformin may be altering proximal insulin signaling in PCa cell line.

3.2 Objectives

a) Establishment of PCa cell line PC-3
b) To study the effect of concentration and contact periods of insulin on PCa cell line
c) To study the effect of concentration and contact periods of metformin on PCa cell line
d) To study the effect of concentration and contact periods of metformin, and insulin combination on PCa cell line

3.3 Rationale

There are controversial divergent health views of the diabetes and PCa. Several Epidemiological studies reported that diabetes decreases the risk of PCa (Darbinian et al. 2008, and Velicer et al. 2006) and numerous epidemiological studies show a positive relationship between metabolic syndrome and cancer. The effect of insulin and antiproliferative activity of metformin on PCa needs to be studied more thoroughly. Meta-analyses also clarify positive association between and PCa, metabolic syndrome and diabetes. In vitro studies of insulin, metformin and PCa cell line would help to study the mechanism involved in the insulin and insulin growth factor signaling which provides more accurate picture regarding link between metabolic syndrome and cancer.

CHAPTER – IV

4. METHODOLOGY AND TECHNIQUES

4.1. Selection of Subjects

Immortalized Cell line- An immortalized cell line is an in vitro clone of mutated cells that can divide indefinitely by evading cellular senescence mechanisms in contrast to the regular cells which can only divide approximately 50 times. There are three out of 21 established cell lines, viz., PC3, DU145 and LNCaP that are routinely used in PCa research. However, these are all derived from metastases and therefore do not have all of the original PCa phenotypes.

4.2. Experimental Models and Study Design

4.2.1. PC-3 cell line experimental model

PC-3 cell line is an androgen insensitive, p53 negative and Kirsten-Ras muted human PCa cell line. These unique characteristics make this cell line less prone to the interference of androgens including testosterone, tumor suppression and Ras gene activation. PC-3 cells have higher metastatic potential compared to DU145 cells. This cell line expresses PSA (Prostate Specific Antigen).

4.2.2. Cell culturing and sub culturing

PC-3 human PCa cell lines were gifted from NCCS, Pune. PC3 cells were grown in Ham-12 media (Himedia) containing 10% fetal bovine serum (invitrogen) and 1% penicillin/streptomycin (Himedia) and have to be maintain in a 37 Centigrade incubator in a 5% CO2 humidified atmosphere. When the cells are about 70% confluent ,remove and discard culture medium than rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA (Himedia) solution to remove all traces of serum that contains trypsin inhibitor. Add 2.0 ml of Trypsin-EDTA solution to flask incubate until cell layer is dispersed (8minutes). Add 6.0 to 8.0 ml of Ham-12 containing 10% FBS medium and 1% penicillin/streptomycin. Add appropriate

aliquots of the cell suspension to new culture vessels and have to be maintain in a 37 Centigrade incubator in a 5% CO2 humidified atmosphere.

4.3. Insulin treatment

10,000 cells per well seeded in 96 well plate media containing 10% fetal bovine serum and 1% penicillin/streptomycin after 24 hours incubation remove the media and added FBS free media serum starvation than after 24 hours Cells were treated with different concentration of recombinant insulin (Himedia, 3 dose, 3 time point) and determine the insulin induce cell proliferation by MTT assay (Banday et al. 2005). Work plan using insulin mediated cell proliferation briefly illustrated in Figure 4.

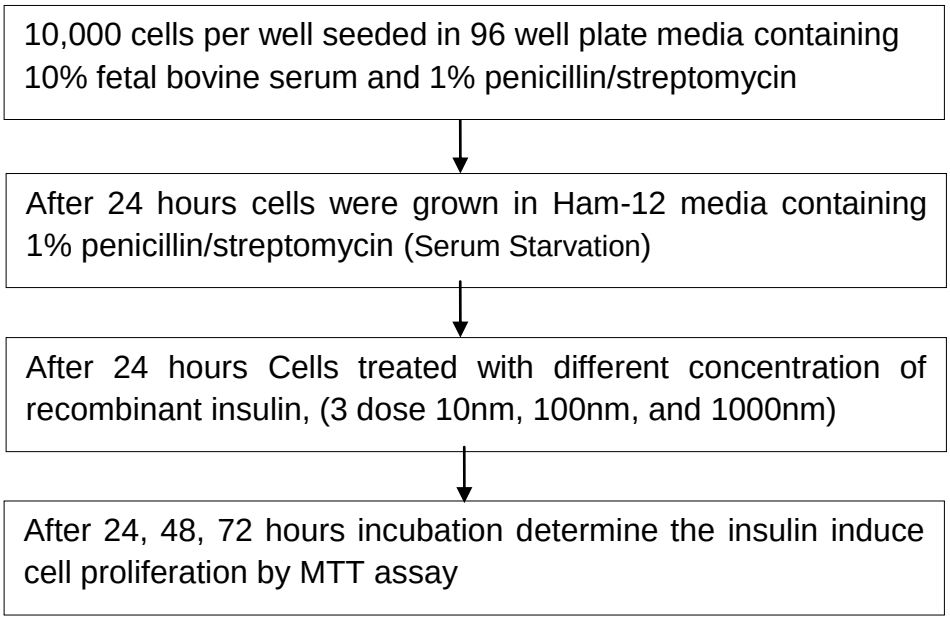


Fig. 4:- Insulin treatment

4.4. Metformin treatment

10,000 cells per well seeded in 96 well plate media containing 10% fetal bovine serum and 1% penicillin/streptomycin after 24 hours incubation remove the media and added FBS free media serum starvation than after 24 hours Cells were treated with different concentration of metformin (3 dose 1µM, 100µM and 1mM) and determine the determine the metformin inhibitive cell proliferation by MTT

assay (Zhuang and Miskimins 2008). Work plan using metformin Mediated Inhibition of Cell Proliferation briefly illustrated in Figure 5.

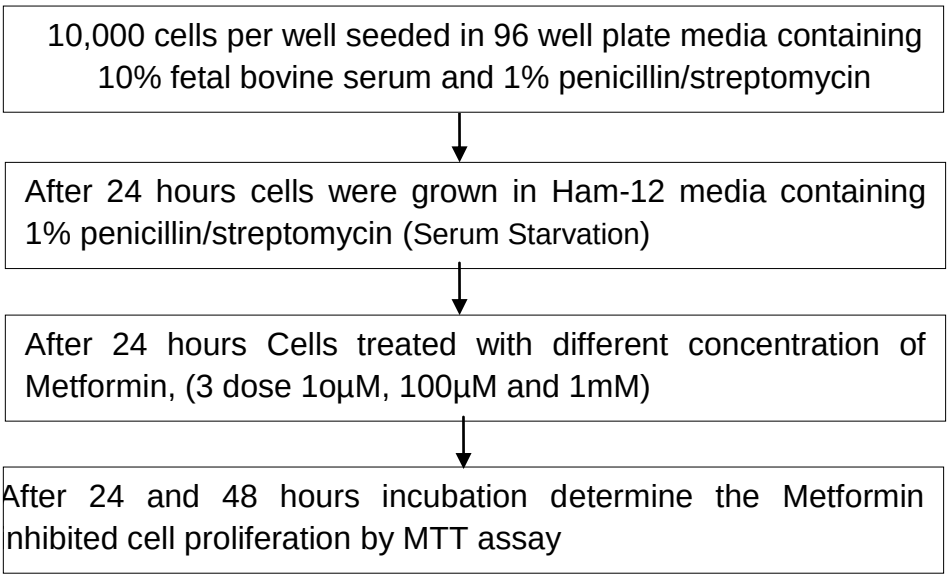


Fig.5:- Cell culturing and metformin treatment

4.5. Effect of metformin on insulin mediated cell proliferation

10,000 cells per well seeded in 96 well plate media containing 10% fetal bovine serum and 1% penicillin/streptomycin after 24 hours incubation remove the media and added FBS free media serum starvation than after 24 hours Cells were treated with different concentration of metformin (3 dose 1μM, 100μM and 1mM) and insulin 100nM and determine the Effect of metformin on Insulin Mediated Cell Proliferation by MTT assay (Zhuang and Miskimins 2008). Work plan using Effect of metformin on Insulin Mediated Cell Proliferation briefly illustrated in Figure 6.

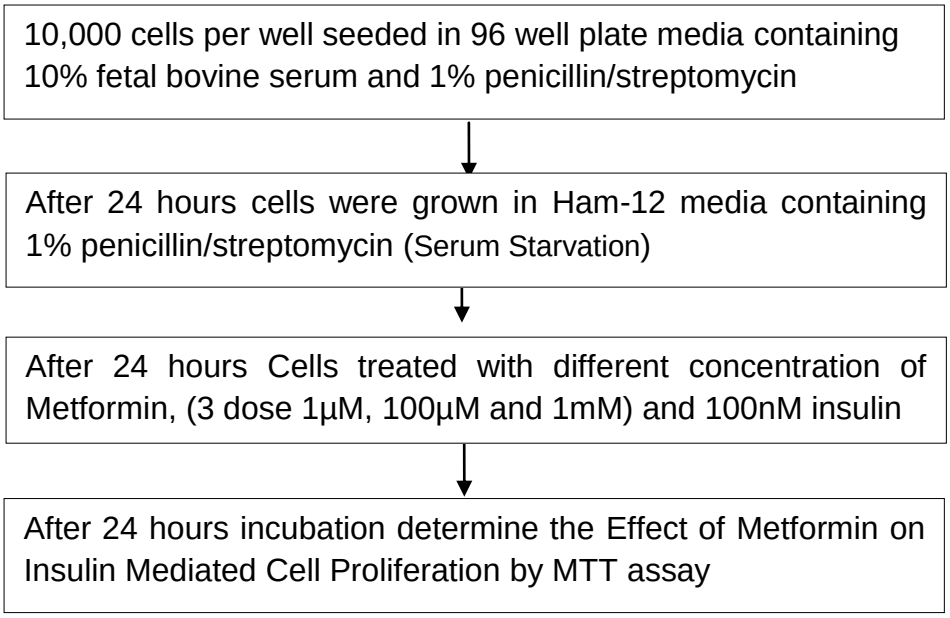


Fig. 6:- Effect of metformin on insulin mediated cell proliferation

4.6. Techniques

4.6.1. Hemocytometer

The hemocytometer is commonly used to determine the concentration of cells in a cell suspension. Cell culture was maintained in Ham-12 with FBS and after trypsinization, cells were added to a Ham-12 complete media. 10µl cell suspension loaded in hemocytometer. The cells were visualized on an inverted microscope and the cells in four 16 quadrate were counted. An average in these four 16 quadrate were taken and the actual cell number present in cell suspension was thus determined.

4.6.3. MTT Assay

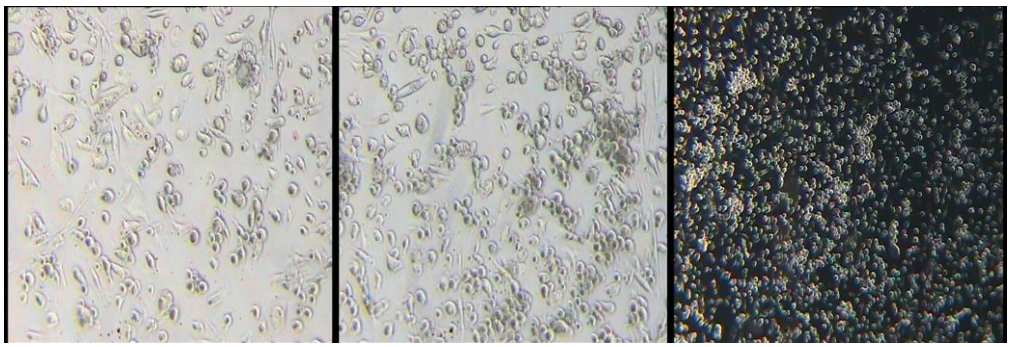
The yellow tetrazolium salt MTT, (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color can then be quantified by spectrophotometric reading at 570 nm. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. 10,000 per well PC3 cells were seeded in Ham-12 media containing 10% fetal bovine serum and 1% penicillin/streptomycin. After 24 hours Ham-12 media without FBS incubation for 24 hours (serum starvation) and then treated with different concentration of insulin and metformin. MTT cell proliferation assay were performed as per the protocol by (Papageorgiou et al. 2008). MTT absorbance was measured at 570 nm and the graph between cell number and dose of treatment was plotted.

Chapter – V

5. RESULTS

5.1. Establishment of PCa Cell Line PC-3

PCa cell line PC-3 cell line is a bone metastasis of a grade IV prostatic adenocarcinoma originated from 62-year-old male Caucasian. These cells exhibit low acid phosphatase and testosterone-5-alpha reductase activities. PC-3 cell line is an androgen insensitive, p53 negative and Kirsten-Ras mutated human PCa cell line. These unique characteristics make this cell line less prone to the interference of androgens including testosterone, tumor suppression and Ras gene activation.



A B C

Fig.7:- PC-3 cell line. A and B shows 10X inverted microscope image of confluent PC-3 cultured flusk. C shows 40X inverted microscope image of confluent PC-3 cultured flusk.

PC-3 human PCa cell line was procured from NCCS, Pune. PC-3 cells were grown in Ham-12 media containing 10% fetal bovine serum and 1% penicillin/streptomycin and have to be maintain in a 37 Centigrade incubator in a 5% CO2 humidified atmosphere.

5.2. Dose and time dependent effects of human recombinant insulin on PC-3 cell metabolism and growth.

Human PCa PC-3 cell line responded to insulin. Human PCa PC-3 cell line treated with insulin concentrations of 10nM, 100nM and 1000nM. Insulin stimulates the cells and increases the cell proliferation dose and time dependent manners. The dose and time-dependent effect of human recombinant insulin on PC-3 cell metabolism and growth as assessed by MTT assay. Note that the increasing concentrations of the human recombinant insulin 10nM, 100nM and 1000nM resulted in a dose-dependent activation of the PC-3 cell metabolism and growth after 24 hrs, 48 hrs, and 72hrs incubation with the human recombinant insulin (Fig. 8, 9 and 10).

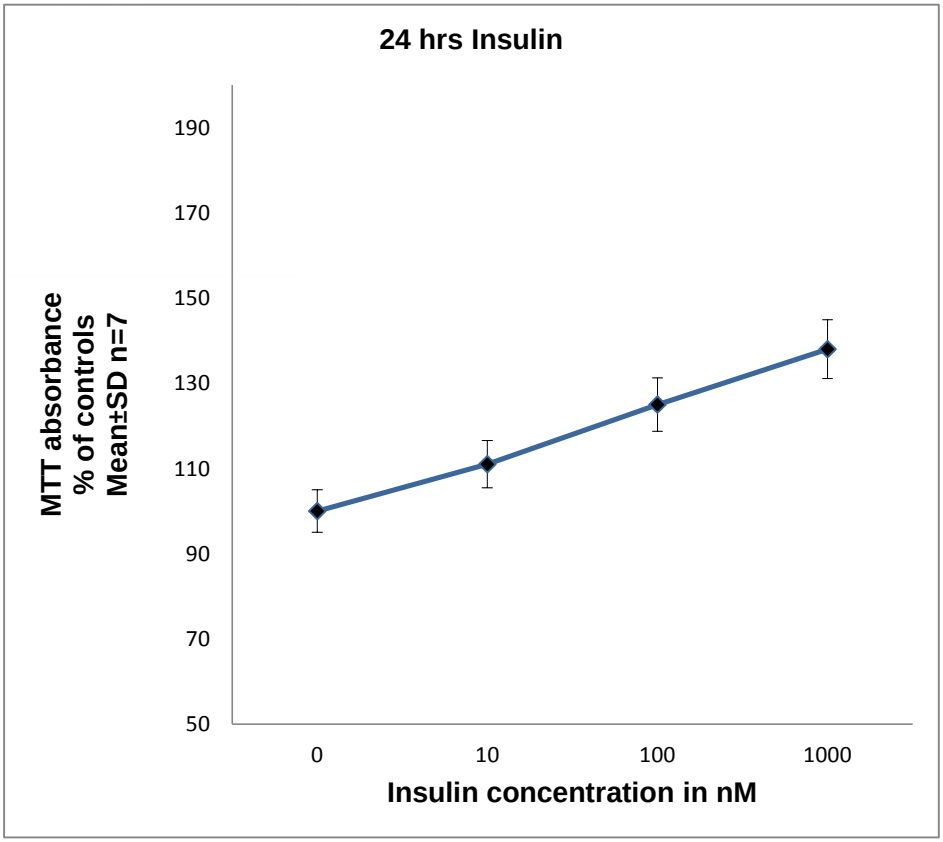


Fig 8:- The dose dependent effect of human recombinant insulin on PC-3 cell after 24 hrs insulin incubation. The human recombinant insulin produced a dose-

dependent activation (10nM, 100nM and 1000nM) of the PC-3 cell metabolism after 24 hrs incubation as assessed by MTT proliferation assays. A 20% increase of the PC-3 cell proliferation was achieved by the concentrations of 100 nM of insulin. Results are expressed as percentage of controls in triplicate experiments. Statistical values are means ± SD ($P < 0.05$).

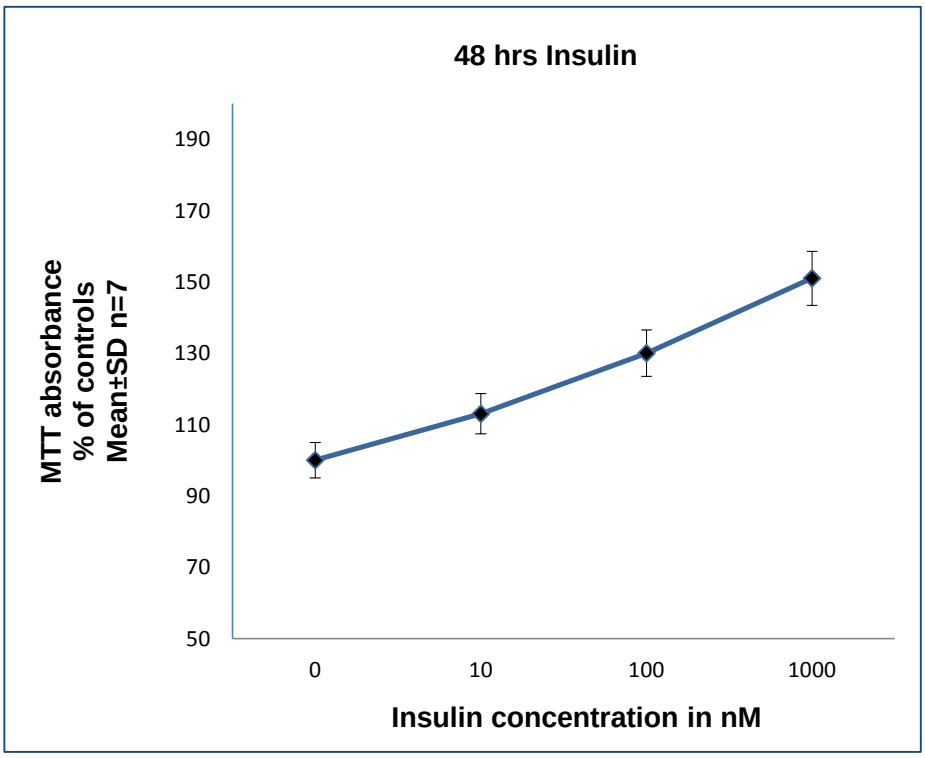


Fig. 9:- The dose dependent effect of human recombinant insulin on PC-3 cell after 48 hrs insulin incubation. The human recombinant insulin produced a dose-dependent activation (10nM, 100nM and 1000nM) of the PC-3 cell metabolism after 48 hrs incubation as assessed by MTT proliferation assays. A 30% increase of the PC-3 cell proliferation was achieved by the concentrations of 100 nM of insulin. Results are expressed as percentage of controls in triplicate experiments. Statistical Values are means ± SD ($P < 0.05$).

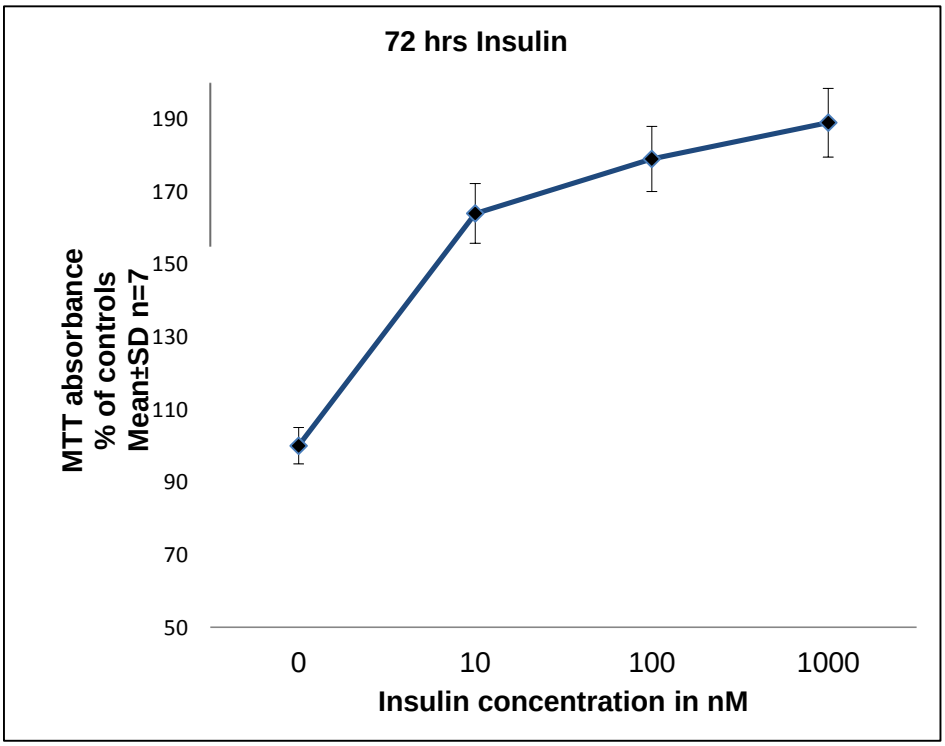


Fig. 10:- The dose dependent effect of human recombinant insulin on PC-3 cell after 72 hrs insulin incubation. The human recombinant insulin produced a dose-dependent activation (10nM, 100nM and 1000nM) of the PC-3 cell metabolism after 72 hrs incubation as assessed by MTT proliferation assays. A 70% increase of the PC-3 cell proliferation was achieved by the concentrations of 100 nM of insulin after 72 hrs incubation. Results are expressed as percentage of controls in triplicate experiments. Statistical Values are means ± SD ($P < 0.05$).

5.3. Metformin inhibits PCa Cell Proliferation.

Insulin and insulin growth factor receptor is important prognostic marker and therapeutic targets for androgen resistance insulin and insulin growth factor associated PCa. To study the effects of metformin in vitro, we used PC-3 cell line. PC-3 cell lines showed growth inhibition with metformin treatment, in a dose dependent manner (Fig. 11 and 12).

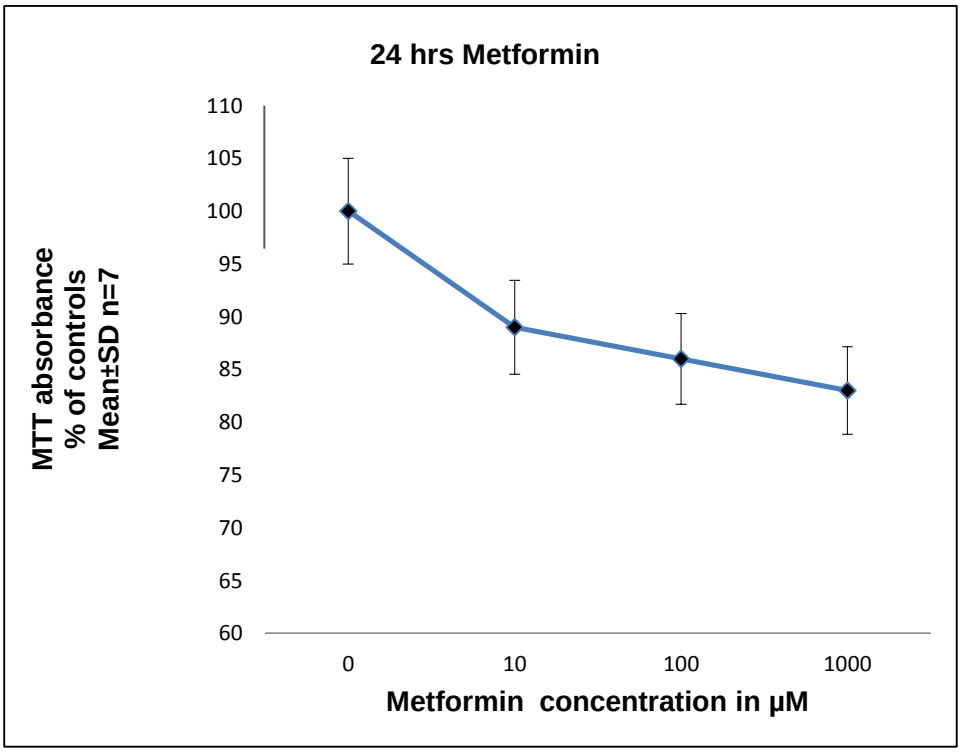


Fig. 11:-The dose dependent effect of metformin on PC-3 cells after 24 hrs metformin incubation. Growth inhibition of PC-3 Cancer cells by the metformin was observed. Cells (10000 per well) were seeded into 96-well plates after 24 hrs cell adherence cell put in 24 hrs serum starvation incubation , than exposed to the indicated concentrations of Metformin , Cell proliferation in each well was measured by MTT assay. A 15% growth inhibition achieved by the concentrations of 100 µM of metformin treatment. Results are expressed as percentage of controls in triplicate experiments. Statistical Values are means ± SD ($P < 0.05$).

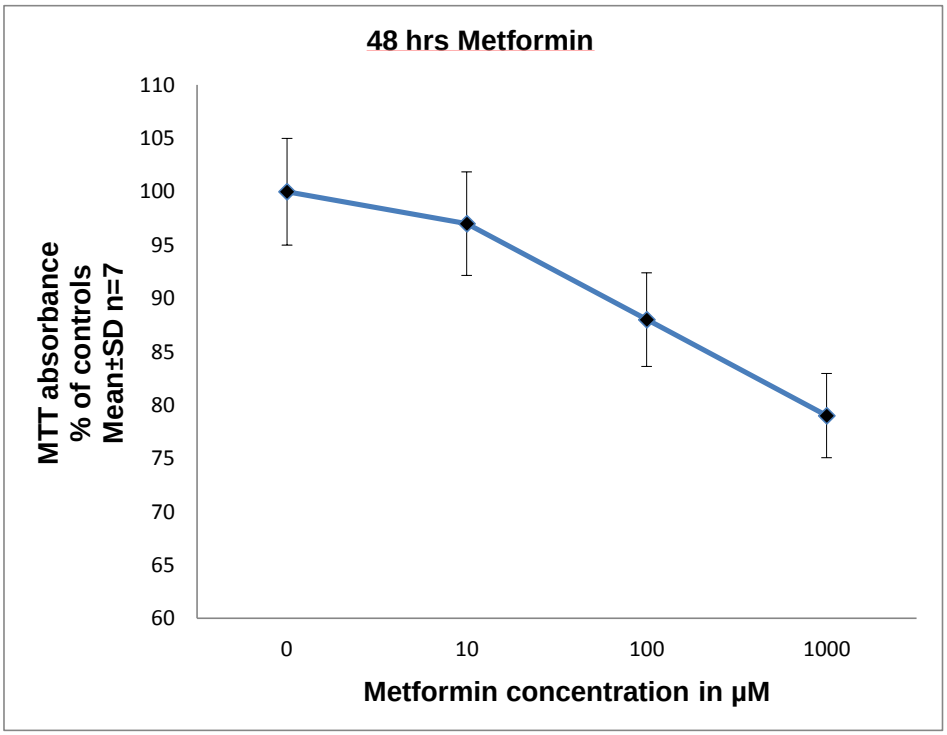


Fig. 12:- The dose dependent effect of Metformin on PC-3 cells after 48 hrs Metformin incubation. Growth inhibition of PC-3 Cancer cells by the Metformin was observed. Cells (10000 per well) were seeded into 96-well plates after 24 hrs cell adherence cell put in 24 hrs serum starvation incubation, than exposed to the indicated concentrations of Metformin, Cell proliferation in each well was measured by MTT assay. A 25% growth inhibition achieved by the concentrations of 1000 µM of metformin treatment. Results are expressed as percentage of controls in triplicate experiments. Statistical Values are means ± SD ($P < 0.05$).

5.4. Metformin acts as a growth inhibitor rather than an insulin inducer for PC-3 human PCa cells.

PC-3 cells are known to be responsive to insulin this is confirmed by the data in Fig. 8, 9 and 10 experiment and growth inhibition with Metformin treatment is confirmed by the data in Fig. 10 and 11 experiment. However, Metformin, rather than enhancing insulin stimulated growth, acted as a growth inhibitor. A 35% cell

proliferation inhibited by combination of 1000µM Metformin and 100nM insulin on PC-3 cell after 24 hrs incubation.

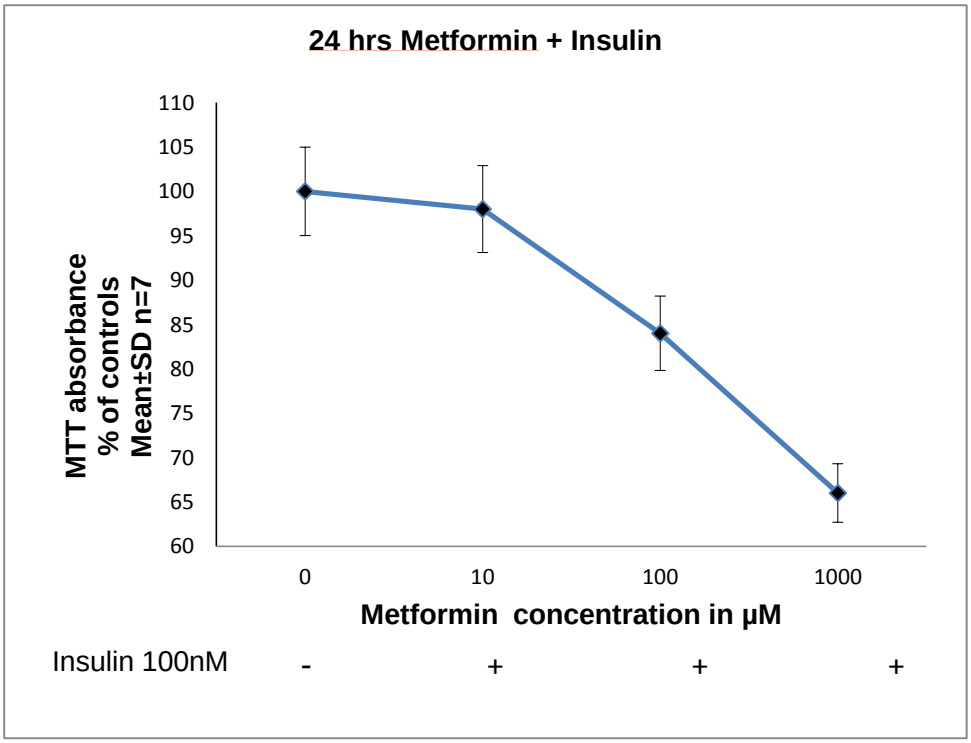


Fig 13:- Effect of Combination Treatments of insulin with Metformin on PC-3 cells after 24 hrs Metformin incubation. The dose and time dependent effects of Metformin (10µM, 100µM, and 1000µM) in combination with a standard dose of human recombinant insulin 100nM on PC-3 cell metabolism and Growth, as assessed by MTT assay. Combination treatment using standard Insulin concentration (100 nM) with increasing concentrations of the Metformin (10µM, 100µM, and 1000µM) has further increased the inhibition of PC-3 cell Growth as assessed by MTT assay. Results are expressed as percentage of controls in triplicate experiments. Statistical Values are means ± SD ($P < 0.05$).

6. DISCUSSION

PCa is the second most frequently diagnosed visceral cancer and the sixth leading cause of cancer death in males. There is evidence that the metabolic syndrome associated with Diabetes type 2 due to the increased insulin levels (Kahn and Flier 2000). Increased insulin levels associated with increased cancer risk worsened cancer prognosis. PCa Growth is influenced by the insulin– IGF signaling axis. There are two mechanisms have been proposed that link between diabetes to the pathogenesis of PCa; these are: Activation of the insulin and insulin-like growth factor associated signaling pathways, and Regulation of endogenous sex hormones. High levels of circulating insulin Increases the risk of PCa initiation and progression. Insulin directly effects the growth, cell proliferation, survival and ultimately leads to resistance to apoptosis of cancer cells. It remains an open question as to whether, or to what extent, differences in circulating levels of insulin influence cancer progression. Insulin effect more severely when patients receiving insulin for the treatment of diabetes, because exogenous insulin increases the risk of PCa initiation and progression. The question whether insulin is capable of inducing or promoting mitogenic effects through its cognate receptor or via the IGF-IR has been a controversial issue for many years. Insulin receptor is expressed in both normal and malignant PCa tissues as Well as in PC-3 lines. Our result highlights the dose dependent effect of human recombinant insulin on PC-3 cell after 24, 48 and 72 hrs insulin incubation. The human recombinant insulin produced a dose-dependent activation (10nM, 100nM and 1000nM) of the PC-3 cell metabolism after 24, 48 and 72 hrs incubation. A 20% increase of the PC-3 cell proliferation was achieved by the concentrations of 100 nM of insulin on 24 hrs incubation and 70% increase of the PC-3 cell proliferation was achieved by the concentrations of 100 nM of insulin on 72 hrs incubation.

Metformin is a widely prescribed oral drug available worldwide at low cost. It reduces glucose levels and enhances insulin sensitivity (or reduces insulin resistance) and it does not result in an increase in circulating insulin hence it does not induce hypoglycemia and hyperinsulinemia. The growth inhibitory effects of metformin are generally by the AMPK dependent as well as AMPK independent.

AMPK is a serine/threonine protein kinase, which serves as an energy sensor in all eukaryotic cell types. AMPK activation leads to G1 cell cycle arrest via up regulation of the p53-p21 tumor suppression gene and ultimately lead to decrease cancer cell proliferation and growth. Metformin did not induce apoptosis during action of cell inhibition (Alimova et al. 2009). As a result, metformin is being investigated as a therapeutic agent in different clinical settings for all PCa subtypes. In this study, we sought to determine the dose and time dependent effect of metformin on PC-3 cells. We found that metformin induced growth inhibitory effects on PC-3 cells.

Our finding highlights the dose dependent effect of metformin on PC-3 cells after 24, 48, and 72 hrs metformin incubation. Growth inhibition of PC-3 Cancer cells by the metformin was observed. A 15% inhibition of the PC-3 cell proliferation was achieved by the concentrations of 1000 µM of Metformin on 24 hrs incubation and 25% inhibition of the PC-3 cell proliferation was achieved by the concentrations of 1000 µM of metformin on 42 hrs incubation. Our findings also highlight, Combination treatment using standard Insulin concentration (100 nM) with increasing concentrations of the metformin (10µM, 100µM, and 1000µM) has further increased the inhibition of PC-3 cell proliferation.Our findings that the metformin inhibits the proliferation of PCa cells independent of their androgen status but dependent on insulin, suggest that metformin may have broad therapeutic efficacy in PCa treatment. Inhibition of the insulin signaling is gaining attention as a potential new anticancer therapeutic strategy. An important factor insulin and insulin growth factor signaling deregulation contributes the mitogenic activity of insulin, that will influence the efficacy of anti IGFI and receptor tyrosine kinases. It is interesting that metformin acts in several ways that may reduce the effect of insulin associated metabolic changes on PCa cell line. 1) Metformin lowers insulin levels, because insulin can stimulate the cell proliferation of PCa cell line. 2) Metformin may act to increase AMPK activation in cancer cells, resulting in direct growth inhibition. 3) Metformin may act AMPK activation independent in cancer cells which reduces cell proliferation.

7. SUMMARY

This thesis aimed to evaluate the role of IGF axis on PCa initiation and progression. Insulin and insulin-like growth factor type 1 are important modulators of growth and metabolic function in the tissues specific manner and correspondingly, their receptors are abundantly expressed tissue specific. Our result highlights the insulin responsiveness of PC-3 cell line. Insulin increases the cell proliferation of PCa cell line. Many questions pertaining to insulin remain unanswered, especially with regard to their physiological Signaling pathway on the tissue like prostate gland. Metformin is the most widely prescribed drug for the treatment of type 2 diabetes worldwide. Metformin reduces the cell proliferation of PCa cell line. It is interesting that metformin acts in several ways that may reduce the effect of insulin associated metabolic changes on PCa cell line. 1) Metformin lowers insulin levels, because insulin can stimulate the cell proliferation of PCa cell line. 2) Metformin may act to increase AMPK activation in cancer cells, resulting in direct growth inhibition. 3) Metformin may act AMPK independent in cancer cells. Our results raise the attractive possibility for the treatment of PCa by the metformin, a widely used anti type 2 diabetes drugs, it may be a future anti PCa drug.

Future perspective-Understanding the molecular response of metformin in insulin induced cancer will be a new venture in understanding molecular biology of cancer. Investigating the up and down regulated insulin receptor and insulin growth factor receptor signaling molecules in insulin induced cancer could plausibly re-write the new treatment target and provide more rational approach to treat the diabetic PCa.

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