TO STUDY THE DOSE AND TIME DEPENDENT EFFECT OF HUMAN INSULIN AND METFORMIN ON THE GROWTH OF BREAST CANCER CELLS

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BY

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CERTIFICATE

I declare that the thesis/dissertation entitled **"TO STUDY THE DOSE AND TIME DEPENDENT EFFECT OF HUMAN INSULIN AND METFORMIN ON THE GROWTH OF BREAST CANCER CELLS"** has been prepared by me under the guidance of Dr. Sanjeev K. Thakur, Assistant Professor, Centre for Bioscience, Central University of Punjab, Bathinda. No part of this thesis/dissertation has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

To Study the Dose and Time Dependent Effect of Human Insulin and Metformin on the Growth of Breast Cancer Cells

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Cancer and diabetes, both are leading causes of mortality globally. Both the diseases are multifactorial and share number of common risk factors. Hyperglycemia and hyperinsulinemia which are the characteristic features of diabetes influences the growth rate and proliferation of tumor cells directly or indirectly. Type 2 diabetes shows stronger association with various cancers. Breast cancer is one of the malignancy affecting females worldwide. This study demonstrates that glucose not only acts as energy source in tumor cell but also acts as mitogen. Insulin not only regulates the blood glucose level but also induces growth and proliferation in MCF 7 and MDA MB 231 breast cancer cell lines independently and in combination with glucose. Metformin inhibit proliferation of MCF 7 and MDA MB 231 breast cancer cell lines independently and also in presence of glucose and insulin, but shows more inhibitory effect in presence of insulin as compare to glucose. Recently discovered insulin receptor antagonist S961 did not showed any significant response in breast cancer cell lines MCF 7 and MDA MB 231. The ineffectiveness is probably due to blocking effect of higher insulin dose. So with this investigation it can be concluded that metabolic alteration leads to proliferation of breast cancer cell lines.

(Ravi Parkash Cholia) (Dr. Sanjeev K. Thakur)

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(Ravi Parkash Cholia)

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1.	AMP activated protein kinase	AMPK
2.	Breast Cancer	BC
3.	Degree Celsius	°C
4.	Dulbeccoôs modified eagles medium	DMEM
5.	Cyclin dependent kinases	CDKs
6.	Extracellular signal related kinase	ERK
7.	Estrogen receptor	ER
8.	Estrogen receptor positive	ER+ve
9.	Fetal bovine serum	FBS
10.	Glycogen synthase kinase 3ß	GSK 3ß
11.	Human epidermal growth factor 2	HER-2
12.	Insulin receptor	IR
13.	Insulin like growth factor I	IGF I
14.	Insulin like growth factor II	IGF II
15.	Insulin receptor substrate	IRS
16.	Leukocyte antigen related tyrosine kinase	LAR
17.	Liver kinase B1	LKB1
18.	Mitogen activated protein kinase	MAPK
19.	Mammalian target of rapamycin	mTOR
20.	Microlitre	µL

21.	Micromolar	µM
22.	Molar	M
23.	Millilitre	mL
24.	Millimolar	mM
25.	(3-(4, 5-dimethylthiazol-2-y1)2, 5-diphenyl tetrazolium bromide)	MTT
26.	Nanomolar	nM
27.	Phosphate buffered saline	PBS
28.	Phosphoinositide dependent kinase	PDK
29.	Phosphatidylinositol-3-kinase/Phosphoinositide-3-kinase	PI3K
30.	Phoshatidylinositol-3-phosphate	PIP3
31.	Protein kinase B	PKB
32.	Protein kinase C	PKC
33.	Progesterone receptor	PR
34.	Progesterone receptor positive	PR+ve
35.	Sex hormone binding globulin	SHBG
36.	Src homology-2	SH2
37.	Type 2 diabetes	T2D

CHAPTER 1

INTRODUCTION

Introduction

Cell divide through a series of coordinated events involving DNA replication and is mainly controlled by signaling cascades and a set of checkpoint proteins known as cyclin dependent kinases (Collins *et al.*, 1997; Nigg, 2001). These signaling events are tightly regulated in normal cells thus enabling them to switch between proliferative and quiescent state whereas these controlling mechanism are defective in abnormally proliferating/malignant/cancer cells (Pardee, 1974). Dysregulated activity of cyclin dependent kinases (CDks), which induce unscheduled proliferation and genome instability (Malumbres & Barbacid, 2009) aided by error in replication, repair and segregation of genome (Hartwell & Kastan, 1994) ultimately results into cells with tumorigenic potential.

1.1 Cancer

Cancer is one of the most complex disorder characterized by abnormal cell growth and clonal expansion having variable genetic, karyotypic or phenotypic trait (Plankar *et al.*, 2011), poorly differentiated or undifferentiated (Hameroff, 2004) and weaker adhere ability (Coman, 1944). Hippocrates, a Greek physician used the terms *carcinos* (a tumor), *carcinoma* (a malignant tumor), and *cancer* (a non-healing malignant ulcer) for abnormally growing cells (Hajdu, 2004). Tumor cells shows great heterogeneity within the same tumor (Hameroff, 2004; Plankar, et al., 2011) and divide through mitotic division as normal cells of the human body but acquire mutation in DNA sequence or acquire new DNA sequences from exogenous sources like viruses such as human papilloma virus, epstein barr virus, hepatitis B virus, human T lymphotrophic virus 1 and human herpes virus 8. Normal cell DNA is continuously damaged by internal and external mutagens, most are repaired by DNA repair system of cell, but small fraction fixes due to low error rate of DNA replication (Stratton *et al.*, 2009), reactive oxidants, malfunction in DNA duplication and repair machinery increases the incidences of random mutations (Hameroff, 2004). Genetic alteration which leads to development of cancer is gain of function mutation such as activation of oncogene and loss of function mutation such as mutation in tumor suppressor gene (Collins, et al., 1997). Two kinds of genes, tumor suppressor genes which act as brake for cell division and oncogenes which are accelerator of cell division, play important role in cancer development. Mutation in the mitotic checkpoint genes found in the tumor cells leads to genetic

instability and serve as fuel for cancer progression (Plankar, et al., 2011). Tumor cells synthesize and respond to own growth factors, which support their development (Sporn & Roberts, 1985). Over production of growth factor linked to the tumor development (Cross & Dexter, 1991). For proper nutrition and oxygen supply angiogenesis, formation of new blood vessels from existing vessels and arteriorgenesis, remodeling of existing artery are the basic necessity for the tumor development (Semenza, 2007). Angiogenesis is the key step for the tumor growth and metastasis (Patenaude *et al.*, 2010). Cancer cell spread to distant site via a complex, multistep process called metastasis (Li *et al.*, 2006). For this cancer cell first lose their adherent ability with adjacent tumor cells and gain migratory and invasive capability by changing gene expression and function, migrate to other site via blood vessels and invade other tissues results in metastasis which causes 90% deaths due to cancer (Yilmaz *et al.*, 2007).

1.2 Diabetes

Diabetes is a group of metabolic disorder mainly characterized by elevated glucose levels. Type 1 and type 2 diabetes are two main forms of diabetes (Malecki, 2005). Type 1 diabetes is due to autoimmune attack on pancreatic ɓ islet cells (Heras *et al.*, 2010) and characterized by ketoacidosis (Hirai *et al.*, 2002). It account for 5-10 % of total diabetic patient (Vigneri *et al.*, 2009) whereas type 2 diabetes is characterized by impairment in insulin secretion, decrease in insulin sensitivity (Malecki, 2005), peripheral insulin resistance, as well as by pancreatic ɓ cell dysfunction (Eldor *et al.*, 2005) and hyperglycemia (Cebioglu *et al.*, 2010). Genetic and environment are the two critical factors in the development of type 2 diabetes (Malecki, 2005). With change in lifestyle, diet, and exercise, treatment with oral antidiabetic agents and insulin, type 2 diabetes can be managed (Warren, 2004). Type 2 diabetes alone account for 90% of all diabetic patient (Vigneri, et al., 2009). Hyperglycemia, excessive urine production, compensatory thirst, increased fluid intake, blurred vision, weight loss and changes in energy metabolism are the consequences of both types of diabetes (Lin & Sun, 2010).

1.3 Relationship Between Cancer and Diabetes

Several studies show that diabetic patients are at higher risk of cancer development. Insulin mitotic signaling is the potential mechanism links between both diseases as insulin used in treatment of diabetes and underlying metabolic abnormalities such as

increased oxidative stress, hyperglycemia, hyperlipidemia, obesity (Chong & Chabner, 2009), overweight, fat rich diet and sedentary lifestyle are the main risk factors behind the development of diabetes (Hussain *et al.*, 2007). Risk for cancer progression is higher in the pre diabetic stage as the insulin level is goes down as diabetes progress (Lipscombe *et al.*, 2006). Insulin signaling pathways, insulin like growth factor signaling pathways and hormonal dysregulation may link both the diseases at cellular level (Wolf *et al.*, 2005).

1.4 Insulin

Insulin is a peptide (Wilcox, 2005), anabolic hormone essential for maintenance of whole body glucose homeostasis, appropriate tissue development and growth of body. It is secreted by ϐ cells of pancreatic islets of langerhans in response to increased circulating level of glucose and amino acid after a meal (Pessin & Saltiel, 2000), maintain blood glucose level, regulate carbohydrate, lipid protein metabolism and promote cell division and growth via its mitogenic effect (Wilcox, 2005). It is used in the treatment of patients with type 1 diabetes as they need insulin treatment permanently and many patients with type 2 diabetes will require insulin as their ϐ cell function declines over time (Migdalis, 2011).

1.5 Metformin

Metformin (1, 1-dimethylbiguanide hydrochloride) is a biguanide commonly used in the treatment of type 2 diabetes mellitus (Zakikhani *et al.*, 2006), low cost oral drug (Alimova *et al.*, 2009), decrease hepatic glucose production (Warren, 2004), lowers circulating insulin levels, decreasing insulin resistance and hyperinsulinemia, so frequently known as insulin sensitizer (Ibarra-Drendall et al., 2011; Zakikhani, et al., 2006). It regulates the AMPK/mTOR pathway, which is involved in protein synthesis and cell proliferation (Ibarra-Drendall, et al., 2011).

CHAPTER 2

REVIEW OF LITRATURE

2.1 Scenario: Diabetes and Cancer

Diabetes is the 12th leading causes of mortality globally (Giovannucci *et al.*, 2010). India, China and the USA are top three countries with maximum number of diabetic patients (Björk, 2001). 25.8 million children and adults are suffered from diabetes out of which 13 million are men and 12.6 million are women aged 20 year or older in United States (ADA, 2012). India has highest number of diabetic patient and referred to as diabetes capital of world (Misra *et al.*, 2011). As documented in the International Diabetes Federation in 2007, India is suffered from 40.9 million diabetic patients which is the highest in worldwide (ID, 2012).

Cancer is the 2nd leading cause of mortality throughout the world (Giovannucci *et al.*, 2010). One in 4 deaths in the United States is due to cancer. As the data from National Cancer Institute, the Centers for Disease Control and Prevention and the North American Association of Central Cancer Registries and mortality data from the National Center for Health Statistics, it has been predicted that a total of 1,638,910 new cancer cases and 577,190 deaths from cancer are projected to occur in the United States in 2012. About 63,300 cases of breast carcinoma *in situ* are expected to be newly diagnosed in 2012. Breast cancer alone is expected to account for 29% (226,870) of all new cancer cases among women. It is estimated that 577,190 Americans will die from cancer this year, corresponding to more than 1,500 deaths per day (Siegel *et al.*, 2012). The total cancer cases in India will likely to go up from 979,786 cases in the year 2010 to 1,148,757 cases in the year 2020. Among males and females, breast cancer alone which is expected to cross the figure of 100,000 by the year 2020 (Takiar *et al.*, 2010). In India it is estimated that there are 2 to 2.5 million cancer patients and about 0.7 million new cases coming every year and nearly half die every year (NCCP, 2012)

2.2 Type 2 Diabetes Mellitus (T2DM)

T2DM is currently the fourth leading cause of death (Cebioglu, et al., 2010). It is a metabolic disorder associated with defects in insulin action on target tissues, skeletal muscle is the primary site of insulin-mediated glucose uptake in human body (Dipl-Pharm, 2005). About 80% patients of T2DM are associated with obesity and sedentary life styles with hyperglycemia due to increased hepatic glucose production,

diminished insulin secretion and impaired insulin action (Lin & Sun, 2010). But men are more prone to diabetes mellitus as compared to the women (Giovannucci, et al., 2010).

2.3 Breast Cancer

Breast cancer (BC) is heterogeneous disease (Martín, 2006), it is nearly sex specific (Giovannucci, et al., 2010), occur in the both male and female but most prevalent in the females (Kabat *et al.*, 2009; Wu *et al.*, 2007). Hormone estrogen and progesterone play important role in the growth, development and physiology of the breast of women (Martín, 2006) and also promote breast cancer proliferation (Pike *et al.*, 1993). There are two major subtypes of breast cancer ductal and lobular carcinomas (Martín, 2006). Estrogen stimulate interlobular ductal cell division while progesterone stimulate terminal duct lobular unit cell division and most breast carcinomas originate from terminal duct lobular unit cells (Martín, 2006; Pike, et al., 1993). BC is categorized on the basis of hormone overexpression or under expression, into different clusters such as luminal A, luminal B, basal like and her2 subtypes. Luminal cancer is the most common subtype. Luminal A has highest expression of ER genes. Basal like breast cancer also called triple negative, is characterized by lack of expression of ER and ER related genes, PR and her2 and expression of cytokeratines 5/6 and 17, EGFR and proliferation-related genes. The majority of breast cancers associated with BRCA1 mutations are basal-like and BRCA2 mutation is luminal A (Martín, 2006). BRCA1 is responsible for the inherited predisposing of breast cancer. The tumor suppressor gene TP53 is found mutated in 25 - 40% of breast cancer cases and in 20% of cases variety of abnormality in retinoblastoma RB1 gene (Lemoine, 1994).

2.4 Type 2 Diabetes Mellitus and Breast Cancer

It is well reported that type 2 diabetes (T2D) increases the cancer risk which can be association with inflammation, insulin resistance, elevated glucose level and the administration of exogenous insulin (Stefansdottir *et al.*, 2011), obesity (Majed *et al.*, 2009), older age (de Miguel-Yanes, 2011; Giovannucci, et al., 2010), unhealthy diet, lack of physical activity (de Miguel-Yanes, 2011; Giovannucci, et al., 2010; Stefansdottir, et al., 2011), alcohol consumption (Giovannucci, et al., 2010), hypertension (Weycker *et al.*, 2009) and genetic history (Mori et al., 2000; Wu, et al., 2007; Xue & Michels, 2007). Age and genetic history are non-modifiable risk factors which cannot be changed and obesity, diet, physical activity, alcohol consumption are

modifiable risk factors which can be changed by changing the life style (Giovannucci, et al., 2010). Diabetic condition induces ~20% greater risk of breast cancer development in diabetic patient (Gallagher & LeRoith, 2010). Type 1 diabetes is weakly associated with cancer (de Miguel-Yanes, 2011). Some earlier studies indicates that hormones dysregulation is the main causes of association between diabetes mellitus and breast cancer (Xue & Michels, 2007). Insulin resistance is associated with postmenopausal and premenopausal breast cancer while the increased estrogen levels is associated with the postmenopausal breast cancer and increased IGFs are associated with the premenopausal breast cancer (Lorincz & Sukumar, 2006; Majed, et al., 2009; Wolf et al., 2006).

2.5 Glucose: Cancer Fuel

A cell requires nutrient and energy for biosynthetic activity of the cell growth and proliferation. Cellular metabolism of tumor cells is influenced by heterogeneity and internal microenvironment within tumor cells. High glucose consumption and lactate secretion rather than oxidizing glucose completely in tumor cell observe first in 1920 by Otto Warburg, a phenomenon called the ôWarburg effectô Glucose metabolism provides various intermediates needed for the biosynthetic pathways such as ribose sugar for nucleotide, glycerol and citrate for lipids, non-essential amino acids and through the oxidative pentose phosphate pathways, NADPH (DeBerardinis *et al.*, 2008). Secreted lactate acidify the tumor environment and providing resistance to acidosis (Masur *et al.*, 2010). Tumor cells have altered metabolic characteristics such as enhanced glucose uptake, aerobic glycolysis, glutaminolysis, nucleic acid synthesis and lipid synthesis and reduced mitochondrial respiration, pyruvate oxidation and acetyl-CoA oxidation as compared to normal proliferating cells (Lyon *et al.*, 1988). Glucose transporter (Glut1 to Glut5) assists the uptake of glucose in human body and tumor cell upregulate and overexpresses Glut1 high affinity glucose transporter (Rastogi *et al.*, 2007). Glucose transport is the first rate limiting step for glucose metabolism (Zhang *et al.*, 2007). Higher glucose concentration induces expression of hypoxia inducing factor (HIF) 1Ŭ which involved in angiogenesis (Dehne *et al.*, 2010). Tumor cells are dependent on glycolysis to support their metabolic requirements. Glucose deprivation induces activation of kinases (mitogen activation protein kinase, C-Jun N terminal kinase, Lyn kinase), change in the redox state of cell or generation of free radicals (Aft *et al.*, 2002).

2.6 Fuel Regulator: Insulin, Insulin Receptor and Signaling

2.6.1 Insulin

Insulin is an anabolic hormone promoting the synthesis and storage of carbohydrates, lipids and inhibiting their degradation and release back into the circulation (Saltiel & Pessin, 2002). It is produced by pancreatic ɓ cells and stimulated by both hormonal and nutritional factors (López-Calderero *et al.*, 2010). Glucose is principle stimulator of insulin secretion. Insulin coded on the short arm of chromosome 11 secreted as its precursor proinsulin, formed by sequential synthesis of signal peptide, B chain, the connecting peptide and then A chain comprising a single chain of 100 amino acids. Active insulin consists of A and B chain linked by disulphide bridges forming of dipeptide containing 51 amino acids with molecular weight of 5802, having isoelectric point at pH 5.5. Chain A comprises of 21 amino acid and B chain 30 amino acids. Two chain are joined by two disulphide bond (Wilcox, 2005). The A chain also has a third internal disulphide bridge. These disulphide bridges hold the molecule together (Mohan, 2002).

2.6.2 Insulin Receptor

Insulin receptor (IR) is a member of tyrosine kinase superfamily (Ward *et al.*, 2007) consists of two extracellular Ŭ subunits and two intracellular ɓ subunits (Dipl-Pharm, 2005), form a $Ŭ_2ɓ_2$ heterotetrameric complex via disulphide bridges (Pessin & Saltiel, 2000). Its coding gene is located on short arm of chromosome 19 (Wilcox, 2005) and more than 150 kilobases in length, contains 22 exons which encode a 4.2 kb cDNA (White, 1997). IR-A and IR-B are two isoforms of IR and IR-A is the predominant IR isoform (Gallagher & LeRoith, 2010; Zhang*, et al.*, 2007). IR-A fragment is 460 bp and IR-B fragment is 636 bp long (Zhang*, et al.*, 2007). IR-B regulate glucose uptake which enhances the metabolic effects of insulin, mainly expressed in adults, in liver, muscle, and adipose tissues whereas IR-A, an exon 11 variant that is expressed in fetal tissues and some tumors (López-Calderero, et al., 2010). IR performs metabolic functions in the normal condition (Blakesley *et al.*, 1996; Lawrence *et al.*, 2007) and also its activation leads to cell proliferation (Zhang*, et al.*, 2007).

2.6.3 Mechanism of Action

Insulin starts its action by binding to its specific transmembrane receptor insulin receptor (IR) (Godsland, 2010). It binds with Ŭ subunit in extracellular domain leads to activation of tyrosine kinase in the intracellular domain, adenosine triphosphate binding and finally receptor autophosphorylation and then insulin receptor substrates (IRS) phosphorylation (Dipl-Pharm, 2005). There are 4 known specifically named IRS proteins, IRS1 to 4. IRS1 mediates the mitogenic action of insulin and major IRS of skeleton muscles. IRS2 in liver mediates peripheral action of insulin and growth of pancreas. IRS3 and 4 are less characterized. IRS3 found only in adipose tissue, fat cells, liver and IRS4 in thymus , brain and kidney (Wilcox, 2005). Other substrates include growth factor receptor bound protein 2 (Grb-2) associated binder-1 (Gab1), p60dok, the c-Cbl proto-oncogene (Cbl), adaptor protein with pleckstrin homology (PH), SIRPS (Saltiel & Pessin, 2002) and Src homology 2 (SH2) domains (Giovannucci, et al.) and 3 isoforms of SH2 domain containing Ŭ$_2$ collagen related protein (Shc). All substrates, except Shc, contain an SH2 domain that targets the substrate to the IR (Dipl-Pharm, 2005). Insulin signaling is transmitted via two major phosphorylation cascades, PI3K (phosphoinositide 3- kinase) and MAPK (mitogen-activated protein kinase) distinguished by their principal mediators (Godsland, 2010). MAPK signaling pathway leads to growth and cell proliferation and PI3K leads to metabolic and antiapoptotic effects (Gallagher & LeRoith, 2010). Protein tyrosine phosphatase (PTPase) PTP1B and LAR have been involved in the negative regulation of insulin receptor by dephosphorylation activity, reduces kinase activity of IR and thereby attenuation insulin action (Pessin & Saltiel, 2000).

PI3K (phosphoinositide 3- kinase) pathway

PI3K involves insulin stimulated glucose uptake (Pessin & Saltiel, 2000) and is a heterodimeric lipid kinase with an important function in the metabolism and mitogenic actions of insulin. The family of PI3-kinase is divided into 3 classes: class I through III PI3-kinase isoforms. PI3-kinase mediate downstream signaling through serine-threonine kinases, 3-phosphoinositide-dependent protein kinase-1 (PDK-1), Akt (protein PKB) and protein kinase Cs (Dipl-Pharm, 2005). Activated Akt1 enhances cell survival, proliferation, growth, apoptosis resistance, inhibit cell spreading and formation and disassembly of adhesive complex (Osborne *et al.*, 2011).

MAPK Pathway

Phosphorylated IRS1 can also mediate the formation of a complex between the adaptor protein Grb2 (growth factor receptor bound protein 2) and the guanine nucleotide-exchange factor mSos (mammalian Son of sevenless). Then complex promote GTP loading and consequent activation of GTPase p21Ras, which activate MAPKs, which then activate transcription factors involved in cell proliferation of different cancers (Godsland, 2010; Osborne*, et al.*, 2011).

2.6.4 IGFs and IGFs Receptors

The insulin-like growth factor (IGF) system is composed of ligands insulin-like growth factor type I (IGF-I), insulin-like growth factor type II (IGF-II), insulin-like growth factor type I receptor (IGF-IR), insulin-like growth factor type II receptor (IGF-IIR) and at least six IGF binding proteins (IGFBPs) which binds to the IGFs (Nahta *et al.*, 2003). Insulin share 49% homology with IGF-I and 61% with IGF-II (Ullrich *et al.*, 1986). They evolved from an ancestral insulin-like gene by duplication (Halper, 2010). Biological functions of both IGF-I and IGF-II are mediated via the IGF-IR (Blakesley, et al., 1996; Daly et al., 1991; Papa et al., 1993; Singer et al., 1995). IGF-IR is a member of tyrosine kinase receptor family like the IR. It is similar to, but distinct from the IR (Papa, et al., 1993). IGF-IR is involved in normal growth, development and differentiation (Blakesley, et al., 1996; Lawrence, et al., 2007; Ward, et al., 2007). The IGF-IIR is a monomeric transmembrane protein that lacks intrinsic signaling activity (López-Calderero, et al., 2010). Major signaling pathways activated by IGF-I are phosphoinositide 3 kinase (PI3K) pathway and the MAP kinase pathway, also known as the ERK pathway (Tosca *et al.*, 2010).

2.6.5 IR/IGFR Axis

The insulin receptor, type 1 insulin-like growth factor receptor and insulin related receptor (IRR) form the insulin receptor family (Lawrence, et al., 2007). Carboxyl termini of the ϐ subunits of the IR and IGF-IR show 44**%** homology and kinase domain shows 85% homology (Faria *et al.*, 1994). Both IR and IGF-IR are heterotetrameric receptors, share high structural homology but differ in specificity (Blakesley*, et al.*, 1996; Lawrence*, et al.*, 2007; Ulanet *et al.*, 2010; Zhang*, et al.*, 2007). The divergent signaling of the IR and IGF-IR is mediated by the ϐ subunit of receptor containing the tyrosine

kinase activity shows distinct preferences for the tyrosine phosporylation domains of IRS. The insulin receptor contains two tyrosine residues whereas the IGF-IR contains three tyrosine residues (Blakesley, et al., 1996). IGF-IR and IR are commonly coexpressed in primary breast cancers and cell lines (Zhang*, et al.*, 2007). IGF-II has higher affinity for the IR-B (Ward, et al., 2007). Insulin is much weaker mitogen than insulin like growth factors (Draznin, 2011). The Ŭ and ɓ chain of IGF-IR and IR can form hybrid receptor by heterotetramerization. The hybrid receptor binds IGF-I and IGF-II with high affinity but has much lower affinity for insulin (Zhang*, et al.*, 2007). Its expression is directly related to IR overexpression in breast cancer (Pandini *et al.*, 1999).

2.6.7 Insulin Receptor Family and Breast Cancer

At cellular level tyrosine kinase domain of insulin shows multiple signaling actions with different effects including change in vesicle trafficking, stimulation of protein kinase and phosphatase, promotion of cellular growth and differentiation and activation or repression of transcription (Saltiel & Pessin, 2002). In hyperinsulinemia, insulin stimulates prenylation of GTPases (Chappell *et al.*, 2001) and expression of leptin, an obesity hormone which promote breast cancer progression (Bartella *et al.*, 2008) via a direct effect on epithelial tissues or indirectly by affecting the levels of insulin-like growth factors (IGFs), adipocytokines (Singer, et al., 1995) and metastases in breast cancer patients (Mahmud, 2008). It stimulate synthesis of IGF-I and IGFBP-3 by up regulating growth hormone (GH) and its signaling in liver and increasing IGF-I bioavailability (Ahmed *et al.*, 2007; Gallagher & LeRoith, 2010; Godsland, 2010) through direct inhibition of IGFBP-1 and IGFBP-2 (Ahmed, et al., 2007). IGFBP-3 induces apoptosis, functions as a negative regulator of breast cancer cell growth (MCF-7) in an IGF independent manner through the activation of caspases involved in a death receptor-mediated pathway (Kim *et al.*, 2004). In breast cancer cells insulin induces aromatase activity and reduces sex hormone binding globulin (SHBG), leading to increase in ovarian estrogen level which increases mitogenic effects (Chowdhury, 2010; Lann & LeRoith, 2008). Estrogen is essential for the growth and development of memory glands (Wolf, et al., 2006; Xue & Michels, 2007). Diabetes Mellitus patient have high circulating level of estrogen (Lipscombe, et al., 2006; Mori, et al., 2000). Hyperinsulinemia elevates estrogen, testosterone, shows mitogenic role in breast

cancer (Larsson *et al.*, 2007) by decreasing SHBG and inducing androgen synthesis in the ovarian stroma (Vigneri, et al., 2009) as shown in figure 2.1.

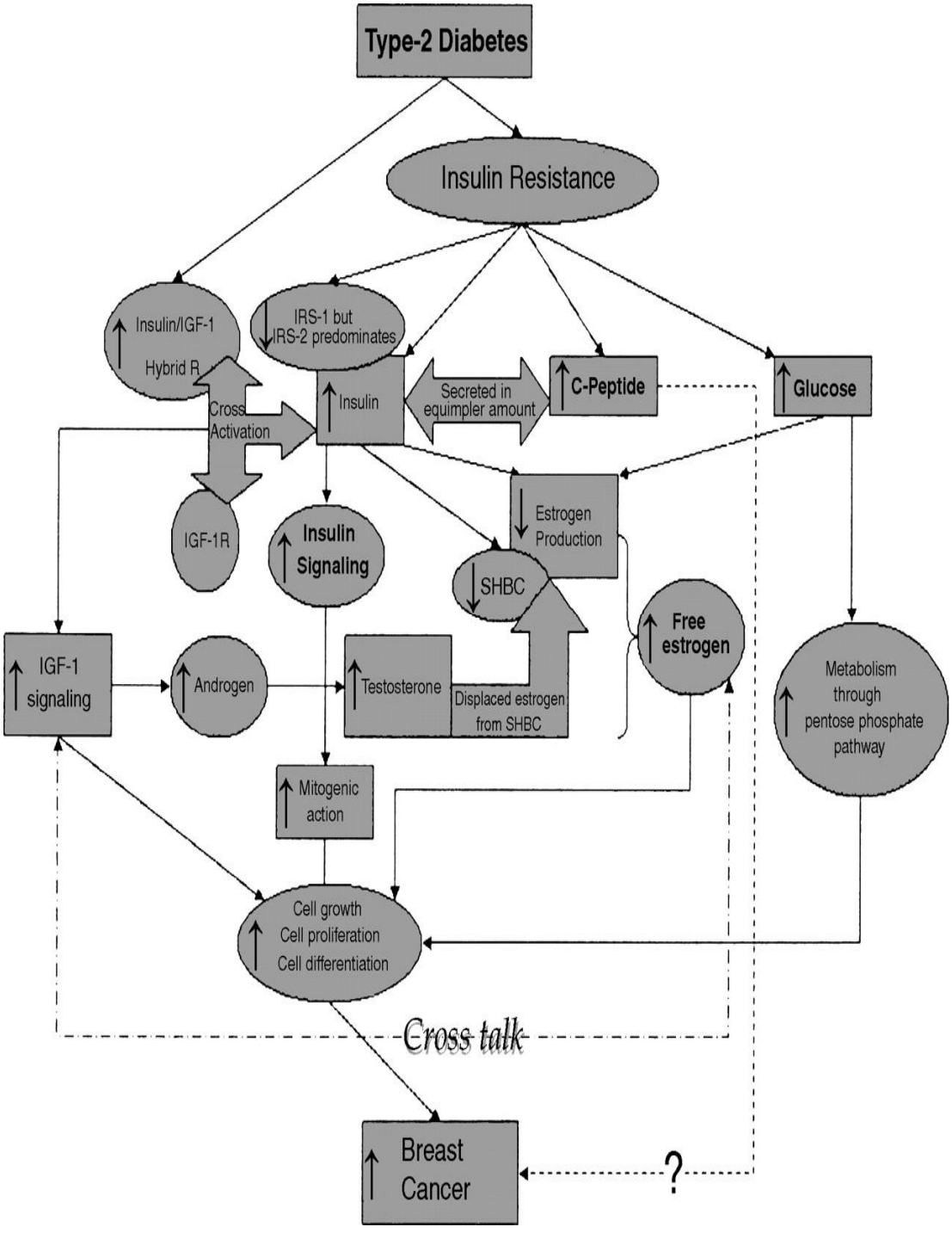

Figure: 2.1 Potential mechanisms for the influence of type 2 diabetes on the risk of breast cancer.
Source: (Xue & Michels, 2007).

2.7 Metformin

Metformin (1, 1-dimethylbiguanide hydrochloride) is a biguanide (Zakikhani, et al., 2006), derived from the herb *Galega officinalis* (French lilac) (Dowling *et al.*, 2011), low cost oral drug available worldwide (Alimova, et al., 2009) commonly used in the treatment of type 2 diabetes mellitus (Zakikhani, et al., 2006), enhances insulin sensitivity without inducing hypoglycemia (Alimova, et al., 2009), increases peripheral glucose utilization and decreases endogenous glucose production by the liver (Phoenix et al., 2009; Tosca, et al., 2010), lowers circulating insulin levels, decreasing insulin resistance and hyperinsulinemia, so frequently known as insulin sensitizer (Ibarra-Drendall, et al., 2011; Zakikhani, et al., 2006). It decreases hyperglycemia and hyperinsulinemia via increases glucose uptake in the skeleton muscles and also shows insulin independent effect through the activation of AMPK. It suppresses the energy consuming processes like gluconeogenesis, protein and fatty acid syntheses (Schott*, et al.*, 2011). It inhibit the hepatic glucose output by inhibiting gluconeogenesis, involves the activation of AMP kinase via an Liver kinase B1 (LKB1) dependent mechanism (Gallagher & LeRoith, 2010; Tosca, et al., 2010; Zakikhani, et al., 2006) which inhibits mTOR pathway, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, increase in the glycolysis and fatty acid oxidation in the breast cancer (Gallagher & LeRoith, 2010). AMPK is activated by a change in AMP:ATP ratio in response to several stimuli, including exercise, hypoxia, hormones (Tosca, et al., 2010), nutrient deprivation, heat shock and metabolic poisoning (Phoenix, et al., 2009). It maintains energy balance by activating the catabolic pathways that generate ATP while inhibiting biosynthetic pathways that consume ATP (Hadad *et al.*, 2009). AMPK is a multisubstrate enzyme (Tosca, et al., 2010) and malfunction of the AMPK pathway undergo uncontrolled proliferation (Hadad, et al., 2009). AMPK is a heterotrimeric consists of one catalytic subunit Ŭ containing a serine/threonine protein kinase and two regulatory subunits ɓ and γ (Phoenix, et al., 2009; Tosca, et al., 2010). Phosphorylation of the Thr172 residue of Ŭ subunit by two known upstream kinases: Liver kinase B1 (Dowling, et al., 2011; Tosca, et al., 2010) and calcium calmodulin dependent kinase kinase (Tosca, et al., 2010). LKB1 is a tumor suppressor and loss of function of LKB1 is associated with Peutz-Jeghers syndrome (Zakikhani, et al., 2006). AMPK pathway induces phosphorylation of TSC2 and regulatory associated protein of mTOR (raptor) which involves in the inhibition of mTOR pathway (Gonzalez-Angulo & Meric-Bernstam,

2010). Metformin also activates AMPK indirectly by disrupting complex I of the mitochondrial respiratory chain, which leads to decreased ATP synthesis and a rise in the cellular AMP:ATP ratio (Dowling, et al., 2011).

Metformin reduces cancer risk and improves cancer prognosis (Zakikhani, et al., 2006). Inhibitory effect of metformin involves activation of AMPK leads to inhibition of mTOR and ribosomal S6 kinase pathway and represses the cell proliferation both in the ER+ve and ER-ve breast cancer cell lines (Phoenix*, et al.*, 2009; Schott*, et al.*, 2011; Zakikhani*, et al.*, 2006) and aromatase expression in adipose stromal cells via activating AMPK. It acts as a growth inhibitor rather than insulin sensitizer in MCF-7 breast cancer cell lines (Schott, et al., 2011). It induces growth inhibition and reduces colony formation through an apoptosis independent mechanism, induces cell cycle arrest at G_1 check point by decreasing cyclin D_1 via upregulation of the p53-$p21^{waf1}$ axis (Alimova, et al., 2009), the reduction of Cyclin D1 levels with the consequent inhibition of the correspondent cyclin dependent kinases, eventually causing an antiproliferative effects by the G1 cell cycle arrest (Cazzaniga *et al.*, 2009).

2.8 Insulin Receptor Antagonist S961

S961 is a novel, biosynthetic, single chain peptide of 43 amino acids, having affinity for the both IR-A and IR-B as insulin but more with IR-A (Schäffer *et al.*, 2008). It is a highly specific peptide insulin receptor antagonist (Vikram & Jena, 2012) causes hyperglycemia, hyperinsulinemia and insulin resistance and depletion of triglyceride and glycogen stores in rats when treated along with insulin combination (Vikram & Jena, 2011).

Aims & Objectives

Human body composed of millions of cells which are coordinated by both internal and external stimulus which may involve genetic as well as environmental factors. Normal functioning of cell is under strict control and regulation. When there is loss of regulation, leads to an alter behavior of cells which may result in bad consequences. Several studies suggest association between two complex disorder diabetes and cancer. This report focused on type 2 diabetes and breast cancer.

The present study carried out with hypothesis that glucose and insulin trigger tumor cell proliferation and metformin inhibit tumor cell proliferation in breast cancer cell lines and explore the relationship between diabetes and breast cancer. The objectives of the study were:

- **Effect of glucose and insulin treatment on progression of breast cancer cell lines.**
- **Effect of metformin treatment and progression of breast cancer cell lines.**
- **Effect of insulin antagonist (S961) in breast cancer prognosis.**

For achieving these objectives this study used MCF 7 and MDA MB 231 breast cancer cell lines.

Cell lines are used in biomedical research as they are assumed to reflect the phonotypical and genotypical characteristics of primary tumor tissue (Tsuji *et al.*, 2010). Breast cancer cell lines have been used widely to investigate breast cancer pathobiology and to screen and characterize new therapeutics (Kao *et al.*, 2009). MCF 7 cell line is first hormone sensitive breast cancer cell line developed (Levenson & Jordan, 1997). Breast cancer cell line MCF 7 is estrogen dependent whereas MDA MB 231 is estrogen independent (Ebert *et al.*, 2011; Yang *et al.*, 2007). MCF 7 is early breast cancer cell line and MDA MB 231 is metastatic cell line (Robey *et al.*, 2005).

CHAPTER 3

MATERIALS AND METHODS

All chemicals and reagents used during experimentation were of cell culture grade.

3.1 Cell Culture

Breast cancer cell lines MCF 7 and MDA MB 231 were obtained from National Center for Cell Sciences (NCCS, Pune, Maharastera, India). Cell lines were cultured in DMEM (HiMedia, India/Gibco, Invitrogen, USA) media supplemented with fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and antibiotics (HiMedia, India / Gibco, Invitrogen, USA).

Cells were cultured at 37^{o}C with 95% humidity and 5% CO_2. Cell lines were maintained and subculture according to their doubling times and incubated in CO_2 incubator (Galaxy 170 R, New Brunswick, Eppendorf, UK). For treatments 5000 cells per well were seeded in 96 well micro titer plate.

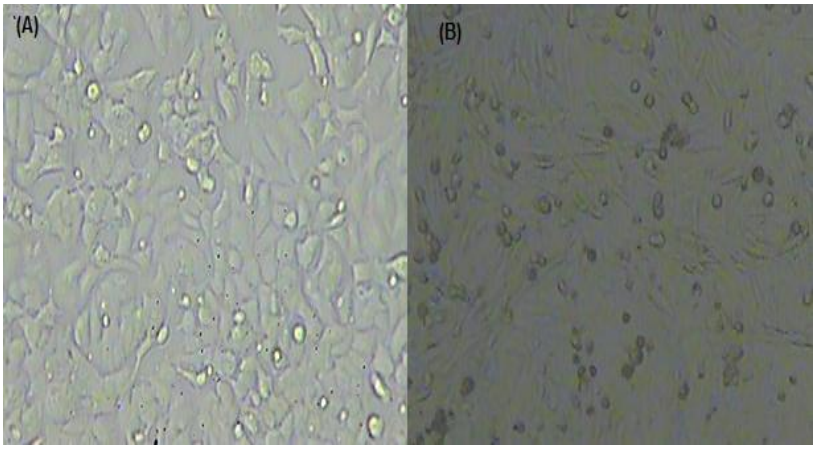

Figure 3.1: *(A) MCF 7 breast cancer cell line and (B) MDA MB 231 breast cancer cell lines*

3.2 Treatments Preparations:

Metformin (free gift from Ranbaxy Laboratories Ltd., India) and Recombinant human Insulin (Biocon, India) were prepared in sterile 1x PBS (HiMedia, India). Glucose (HiMedia, India) stock solution was prepared in sterile water and then autoclaved. All dilutions were done in DMEM media. Lyophilized S961, procured from Novo-Nordisk (Denmark) dissolved in the sterilize PBS and stored at -20^{0}C. Diluted in the DMEM media before use as per the concentration needed (Vikram & Jena, 2012).

4 Treatment Plans

Table 3.1: Treatments Plans

S.No.	Breast cancer cell line	Control	Treatments Groups	Concentration	Incubation period
1.	MCF 7	No Glucose	Glucose	5,10,20,30mM	24, 48, 72h
2.	MDA MB 231	No Glucose	Glucose	5,10, 15, 20, 25 30mM	24, 48, 72h
3.	MCF 7 & MDA MB 231	No Insulin	Insulin	10, 100, 1000, 5000nM	24, 48, 72h
4.	MCF 7 & MDA MB 231	No insulin + normal physiological glucose concentration	Insulin + normal physiological glucose concentration	10, 100, 1000, 5000nM	24, 48, 72h
5.	MCF 7 & MDA MB 231	No insulin + hyperglycemic glucose concentration	Insulin + hyperglycemic glucose concentration	10, 100, 1000, 5000nM	24, 48, 72h
6.	MCF 7 & MDA MB 231	Media only	Metformin	2, 5, 10, 15, 20, 25mM	24, 48, 72h
7.	MCF 7	No glucose	Glucose + Insulin + Metformin	20mM Glucose + 10nM Insulin + 15mM Metformin	24, 48, 72h
8.	MDA MB 231	No glucose	Glucose + Insulin + Metformin	5mM Glucose + 100nM Insulin + 15mM Metformin	24, 48, 72h
9.	MCF 7 & MDA MB 231	Insulin	S961	0.5nM - 100nM	24, 48, 72h
10.	MCF 7	Insulin	S961	10nM	24h

3.5 Cell Growth Assay:

MTT solution was prepared by dissolving in the PBS at 0.5mg/ml concentration and then 100µl per well was added to cell culture micro titer plate. Incubated for 4h at 37^0C, formazan complex formed is solubilized with DMSO (200µl per well) and absorbance measured at 570nm (Aft, et al., 2002) in Microplate reader (Bio Rad, USA).

3.6 Data Analysis:

Data collected was statistically analyzed using SigmaPlot version 11.0. Level of significance was evaluated at P Ò 0.05 using studentôs t-test and for multiple comparisons one way ANOVA was performed with Tukeyôs test.

CHAPTER 4

RESULTS

Results

Since diabetes is associated with proliferation disorder like cancer. We sought to understand the tumorigenic potential of insulin, glucose and antiproliferative activity of antidiabetic drug metformin and role of insulin receptor antagonist S961 in insulin mediated cell proliferation on breast cancer cell lines MCF7 and MDA MB 231. For this cells were treated with varying doses of treatments for different time periods (24h, 48h and 72h) to analyses their effect on cell proliferation.

4.1 Hyperglycemia mediated stimulation of breast cancer cell lines

Glucose is the primary source of energy in the cell which leads to various activities and growth of cell. In tumor cells there is continuous need of nutrition for growth and proliferation. In order to assess the effect of glucose cells were treated in dose and time dependent manner.

4.1.1 MCF-7 Response to Glucose:

The present study indicated that glucose was acting mitogenic in time and dose dependent manner. A significant progressive increase from ~1.3-2.9 folds was obtained with increase in glucose concentration (5-20mM).

Cells showed proliferation even from physiological glucose concentration (5mM) to hyperglycemic glucose concentration (20mM), thereafter, it decreased. Cells were not only responsive to dose but also showed significant change in time dependent treatments. Cells proliferation increased significantly after 24 h and recorded maximum at 48 h, later (72 h) it declined. It can be summarized that glucose showed mitogenic effects even in the physiological concentration (5mM), further increase in concentration lead to further increase of cell proliferation (figure 4.1 A,B,C).

4.1.2 MDA MB 231 Response to Glucose:

MDA MB 231 cells showed a significant ~3 fold increase in cell proliferation in normal physiological glucose concentration (5mM) to the metabolic glucose concentration (10mM) and thereafter decrease in the proliferation rate was observed with further increase in the glucose concentration in 24h (figure 4.1 D). MDA MB 231 was not tested for further increased time dependent treatments.

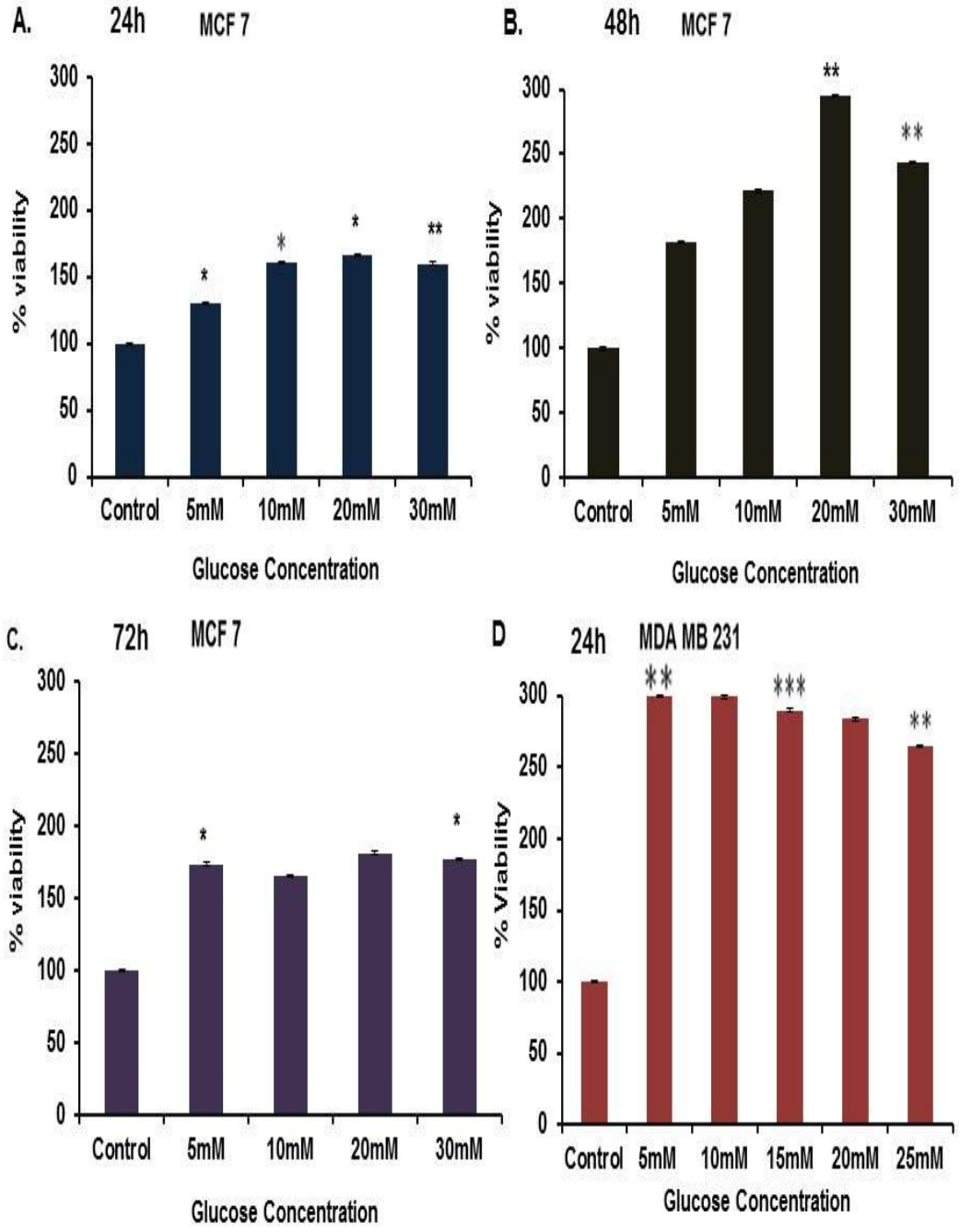

Figure 4.1: Effect of glucose on breast cancer cell lines. *Figure A, B, C showed the effect of glucose in proliferation of MCF 7 cell lines for 24, 48 and 72h respectively and figure D showed MDA MB 231 for 24h. All the values shown as % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukey's test.*

It was quite evident from the present finding that glucose not only acts as source of energy but also act as mitogen for breast cancer cell lines (MCF 7 and MDA MB 231) proliferation. In conclusion both the cell lines respond to glucose treatment and it may be considered as a potential mitogen.

4.2 Insulin induces proliferation in breast cancer cell lines independently and in combination with glucose.

Insulin hormone regulate the blood glucose level in cell and whole body via glucose transporters and involve in metabolic signaling but hyperinsulinemia may alter insulin signaling which leads to cell proliferation.

Since both glucose and insulin showed mitogenic potential we wanted to analyze their synergistic effect on proliferation of breast cancer cell lines. Different concentrations of insulin were tested under normal physiological glucose (5mM) and hyperglycemic glucose concentration (20mM) for 24h, 48h and 72h to check the combined effect.

In present study insulin effect on breast cancer cell lines was investigated in three different experiment, initially only insulin effect then in combination with normal glucose concentration (5mM) and lastly in combination with hyperglycemic glucose concentration (20mM). It was indicated that insulin hormone was acting as mitogen in time and dose dependent manner.

4.2.1 Insulin Induced Proliferation in MCF 7

In 24h insulin treatment, MCF 7 insulin induced ~1.1-2.5 folds significant dose and time dependent cell proliferation from 10nM insulin concentration and increases up to 5000nM with no glucose, but in combination with normal physiological glucose concentration (5mM) showed ~1.8 fold increase up to 100nM insulin concentration and thereafter decrease in cellular proliferation of cells and under hyperglycemic condition it showed ~1.3 fold increase from 10nM to 100nM insulin concentration and thereafter saturation in proliferation of MCF 7 cells on further increases the insulin concentration (figure 4.2 A).

With increase in treatment time to 48h, MCF 7 cells showed ~1.6 fold increase in proliferation rate up to 100nM insulin concentration and there after decrease in proliferation rate was observed when treated with insulin only but under normal

physiological glucose concentration, it snowed ~1.3 fold increases proliferation rate up to 100nM insulin concentration whereas in combination with hyperglycemic glucose concentration (20mM) only ~1.2 fold increase in proliferation was observed with increase in insulin concentration up to 5000nM (figure 4.2 B).

Further increase in treatment time to 72h, insulin did not showed any significant proliferation (figure 4.2 C).

4.2.2 Insulin Induced Proliferation in MDA MB 231

In 24 h treatment, there was increase in the proliferation rate was observed in MDA MB 231 cell line with increase in insulin concentration but in combination with 5mM glucose concentration ~1.2 fold increase in cell proliferation followed by ~1.1 fold in combination with hyperglycemic glucose concentration (20mM) up to 100nM insulin concentration and thereafter decrease was observed with further increase in insulin concentration (figure 4.2 D). In 48h insulin treatment, ~1.6 fold increase in proliferation was observed with increase in insulin concentration from 10nM to 1000nM insulin concentration and thereafter decease in proliferation was observed on further increase in insulin concentration (figure 4.2 E).

In 48h, ~1.8 fold increase in proliferation rate with increase in insulin concentration from 10nM to 100nM in combination with normal physiological glucose concentration (5mM) and thereafter decease in proliferation was observed on further increase in insulin concentration whereas in hyperglycemic glucose concentration (20mM), there was ~1.2 fold increase in proliferation rate was observed only at higher insulin concentration (figure 4.2 E).

In 72h, insulin treatment ~2 fold proliferation rate was observed at 10nM insulin concentration and thereafter decrease in proliferation with further increase in the insulin concentration was observed. While in combination with normal physiological glucose concentration (5mM) there was ~1.6 fold increased proliferation was observed up to 100nM insulin concentration and thereafter decreased and under hyperglycemic (20mM) glucose concentration ~1.9 fold increased proliferation was recorded at initial 10nM insulin concentration and later it decreased with further increase in insulin concentration (figure 4.2 D).

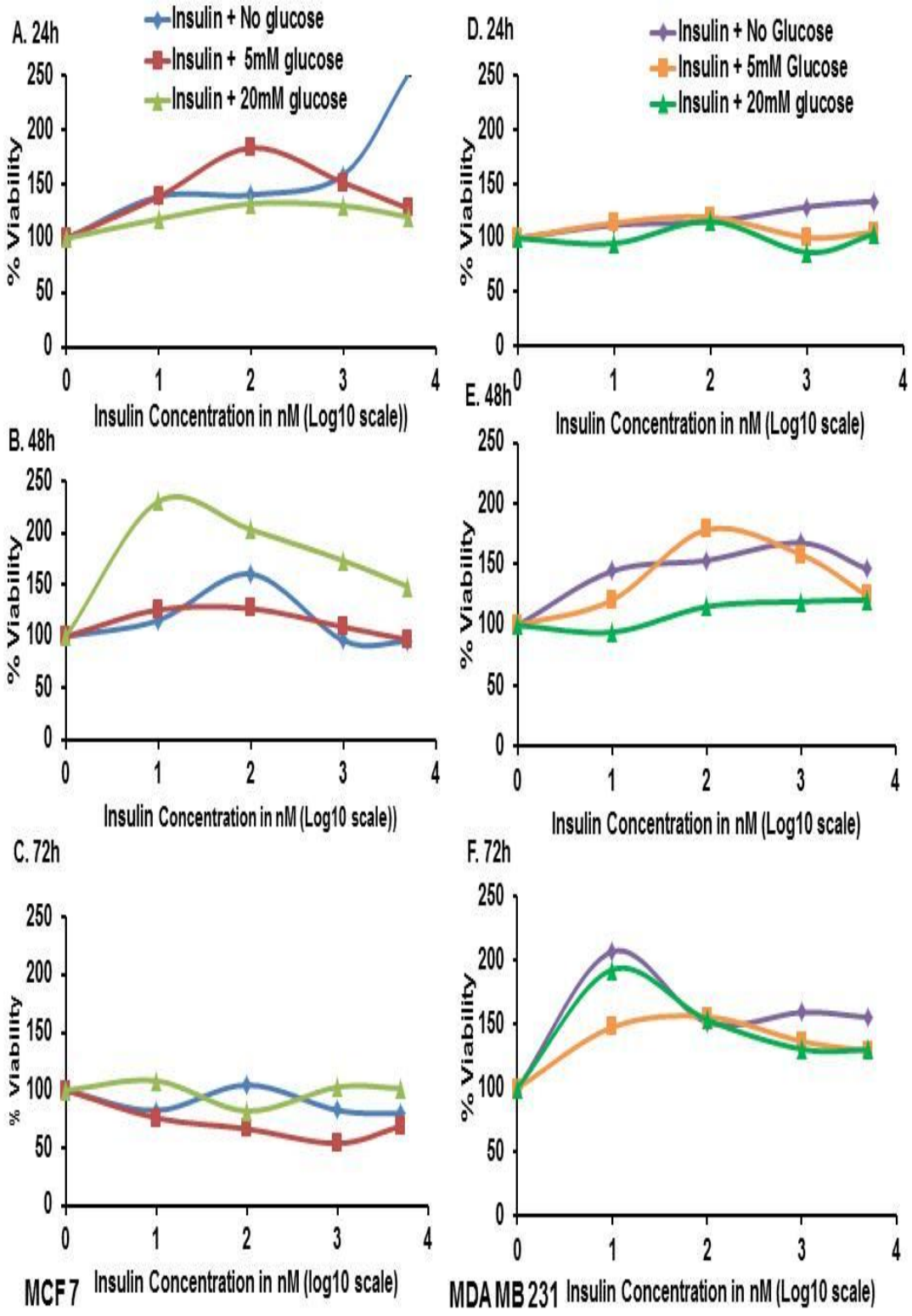

Figure 4.2: Effect of insulin on the proliferation of breast cancer cell lines. *Figure A,B,C showed effect of insulin (0nM, 10nM, 100nM, 1000nM and 5000nM) on the proliferation of MCF 7 and D,E,F: MDA MB 231 for 24, 48, 72h respectively.*

In conclusion insulin showed progressive proliferation only in 24h treatment but no such trend in 48h and 72h and in combination with glucose treatment. Insulin shows proliferative effect in both insulin only treatment and in combination with glucose, clearly indicating that insulin also has glucose independent proliferative action on breast cancer cell lines MCF 7 and MDA MB 231.

4.3 Metformin induces inhibition of proliferation in Breast Cancer Cell Lines

Metformin is widely used in the treatment of type 2 diabetes in controlling the hyperglycemia and hyperinsulinemia. It has been found that this drug is also beneficial in cancer treatment as it shows inhibitory effect in cell proliferation and growth.

The present study tested the antiproleferative properties of metformin in dose and time dependent manner. It starts cell proliferation inhibition after the 2mM metformin concentration but the lower concentration did not showed any inhibitory effect because we studied metformin effect in presence of high glucose concentration (25mM) as in previous experiment it is clearly showed that glucose showed proliferative effect, so due to higher glucose concentration metformin lower concentration effect is suppressed on cell lines.

A significant decrease in proliferation rate ~12 % to ~85 % was observed with increase in metformin concentration (2mM-25mM) in MCF 7 and ~6 % to ~68 % in MDA MB 231 with same metformin concentrations (figure 4.3A,B,C).

Metformin showed inhibition in proliferation rate in both breast cancers cell lines in dose and time dependent treatment though their responsiveness was different. The cell proliferation was significantly inhibited with increase in the concentrations ranging from 2mM to 25mM in both MCF 7 and MDA MB 231 cells at 24h and 48h (figure 4.3 A,B,C).

MCF 7 showed inhibition from 2mM to 25mM in all time period (24h, 48h and 72 h). On the other hand MDA MB 231 cells showed inhibition from 2mM to 25mM in 24h and 48h but there was significant inhibition of proliferation at 15mM in 72h treatment (figure 4.3 C). It was found that 15mM metformin concentration showed ~50% inhibition in proliferation in both the breast cancer cell line.

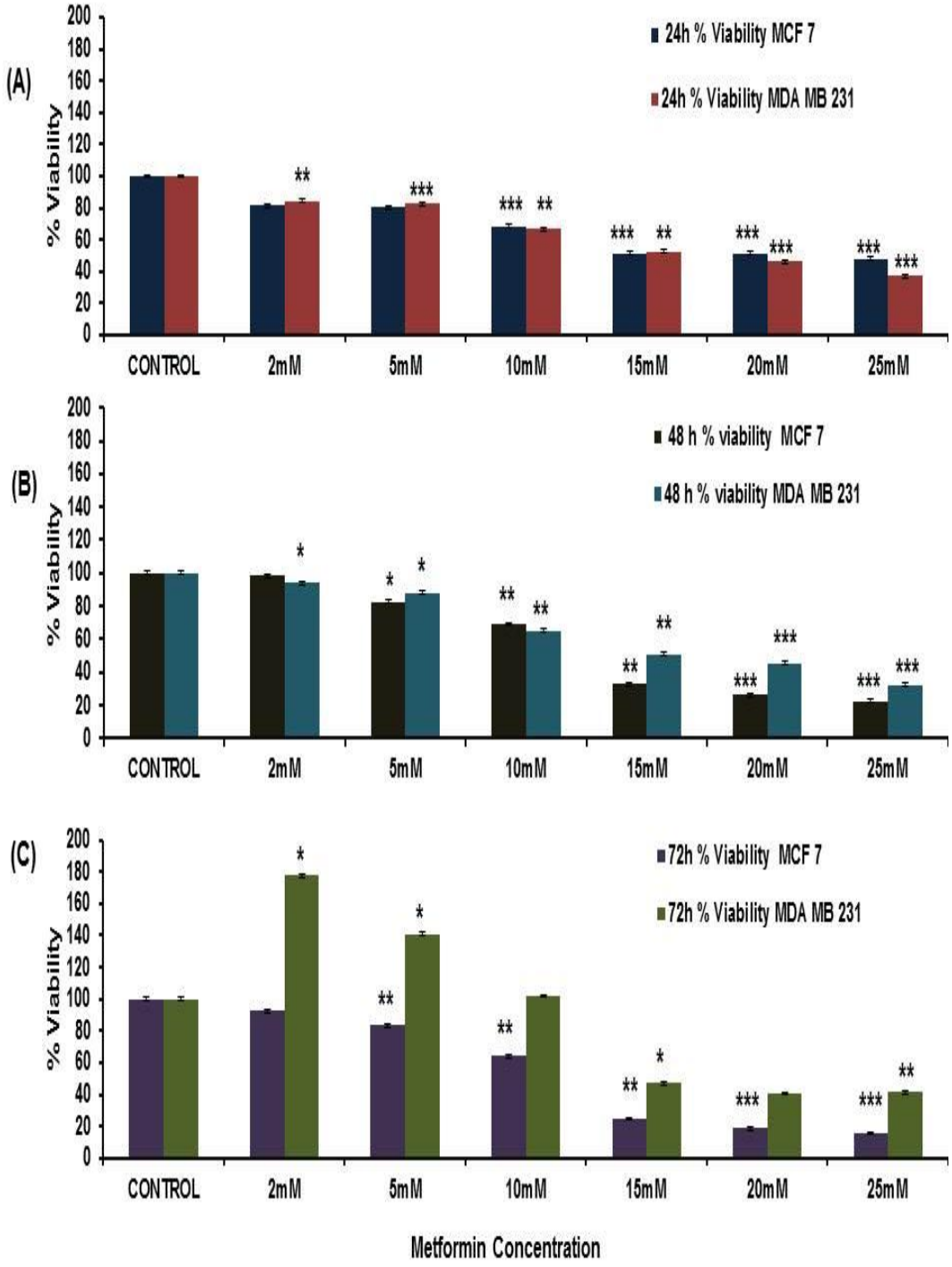

Figure 4.3: Effect of metformin on breast cancer cell lines, MCF 7 and MDA MB 231. *Figure A,B,C showed anti proliferative effect of metformin on MCF 7 and MDA MB 231 for 24, 48, 72h respectively. All the values shown in % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukey's test.*

4.4 Effect of Metformin on the Growth and Proliferation of Breast Cancer Cell Lines in Combination with Glucose and Insulin

Selected dose combination of glucose, insulin and metformin was given to the breast cancer cell lines MCF 7 and MDA MB 231 for 24h, 48h and 72h in order to check the efficiency of the drug under hyperglycemia and hyperinsulinemia conditions. MCF-7 was treated with 15mM metformin in combination with 10nM insulin and 20mM glucose concentration and MDA MB 231 was treated with 15mM metformin in combination with 100nM insulin and 5mM glucose concentration.

When cells were treated with selected dose combination at one stage where insulin with glucose combination showed ~2.5 fold proliferation at the same time metformin when given in combination with insulin and glucose, showed inhibition as compare to untreated cells and maximum in presence of insulin as compared to glucose. Cell proliferation was inhibited by metformin even when given combined with insulin and glucose in both MCF 7 and MDA MB 231 breast cancer cell lines (figure 4.4: A,B,C). It is quite evident from present results that metformin is an effective drug not only in hyperglycemic and hyperinsulinimic conditions but also in breast cancer cells.

4.5 Effect of Insulin Receptor Antagonist S961 on the Growth and Proliferation of Breast Cancer Cell Lines in Combination with Insulin.

Insulin receptor antagonist S961 (0.5nM to 100nM) was tested against 10nM and 100nM insulin concentration in MCF 7 (figure 4.5.1: A,B,C) and MDA MB 231 for 24, 48, and 72h (figure 4.5.2: A,B,C), which did not showed any significant inhibitory effect in both the cell lines.

As the MCF 7 was found more sensitive to Insulin therefore again S961 at 10nM concentration was tested for a range of insulin concentration (0nM ï 100nM) for 24h (figure 4.6 A, B), which again did not showed any significant inhibition in MCF 7 breast cancer cell line.

The present study here concluded with a remark that further testing of S961 at much lower concentrations is required. The ineffectiveness is probably due to blocking effect of higher insulin dose.

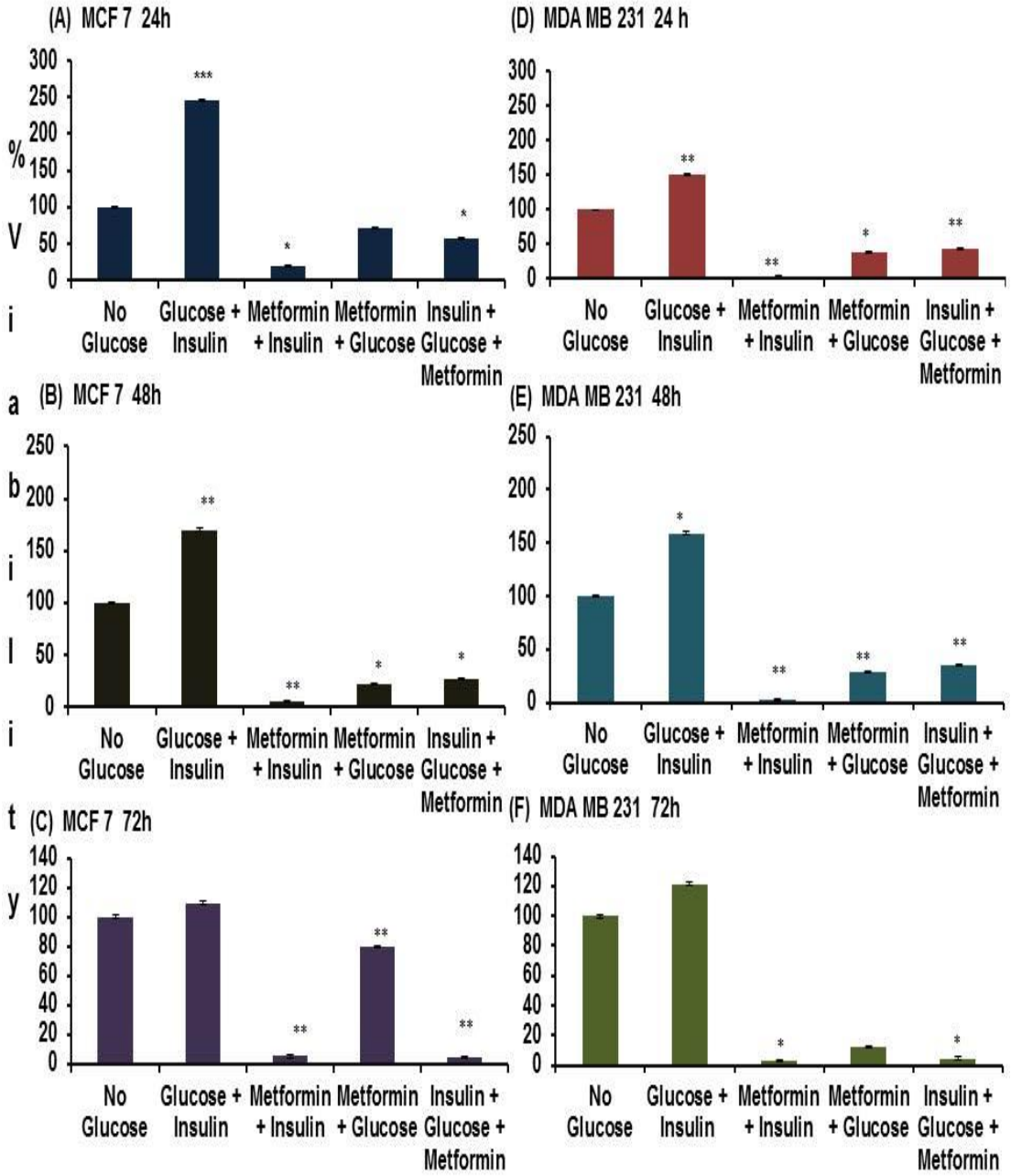

Figure 4.4: Effect of metformin on the breast cancer cells in combination with insulin and glucose. *Figure A,B,C showed effect of metformin (15mM) on MCF 7 in combination with glucose (20mM) and insulin (10nM) for 24h, 48h and 72h respectively and figure D,E,F showed combined inhibitory effect of metformin (15mM) in presence of glucose (5mM) and insulin (100nM) in MDA MB 231 at 24h, 48h, 72h respectively. All the values shown in % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukey's test.*

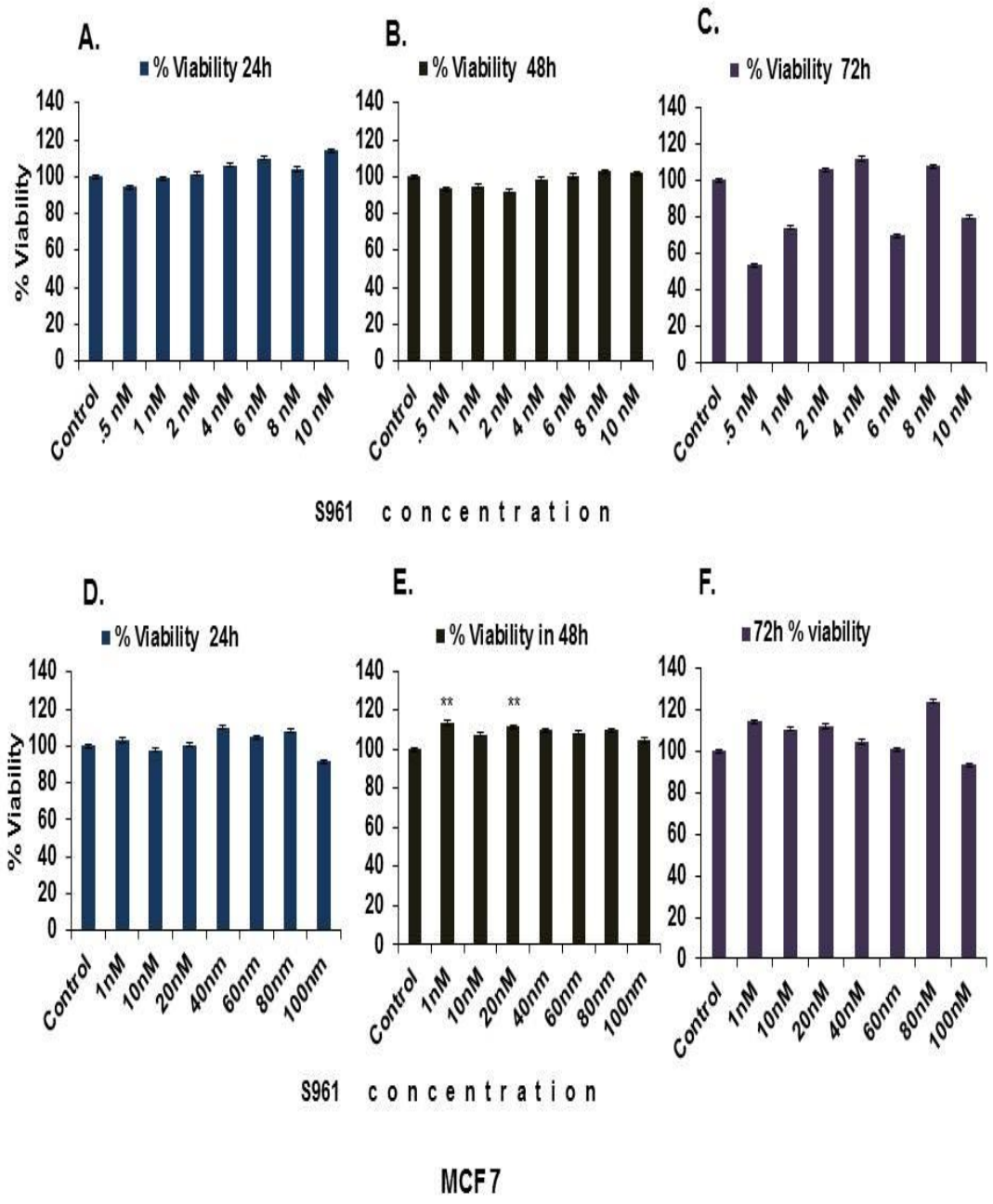

Figure 4.5.1: Effect of insulin receptor antagonist in presence of different insulin concentration on the breast cancer cell line MCF 7. *Figure A, B and C showed the effect of S961 (0.5nM-10nM) in combination with 10nM insulin concentration and figure D, E and F showed the effect S961 (1nm-100nM) in combination with 10nM insulin concentration for 24, 48 and 72h respectively in MCF 7 breast cancer cell lines. All the values shown in % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukey's test.*

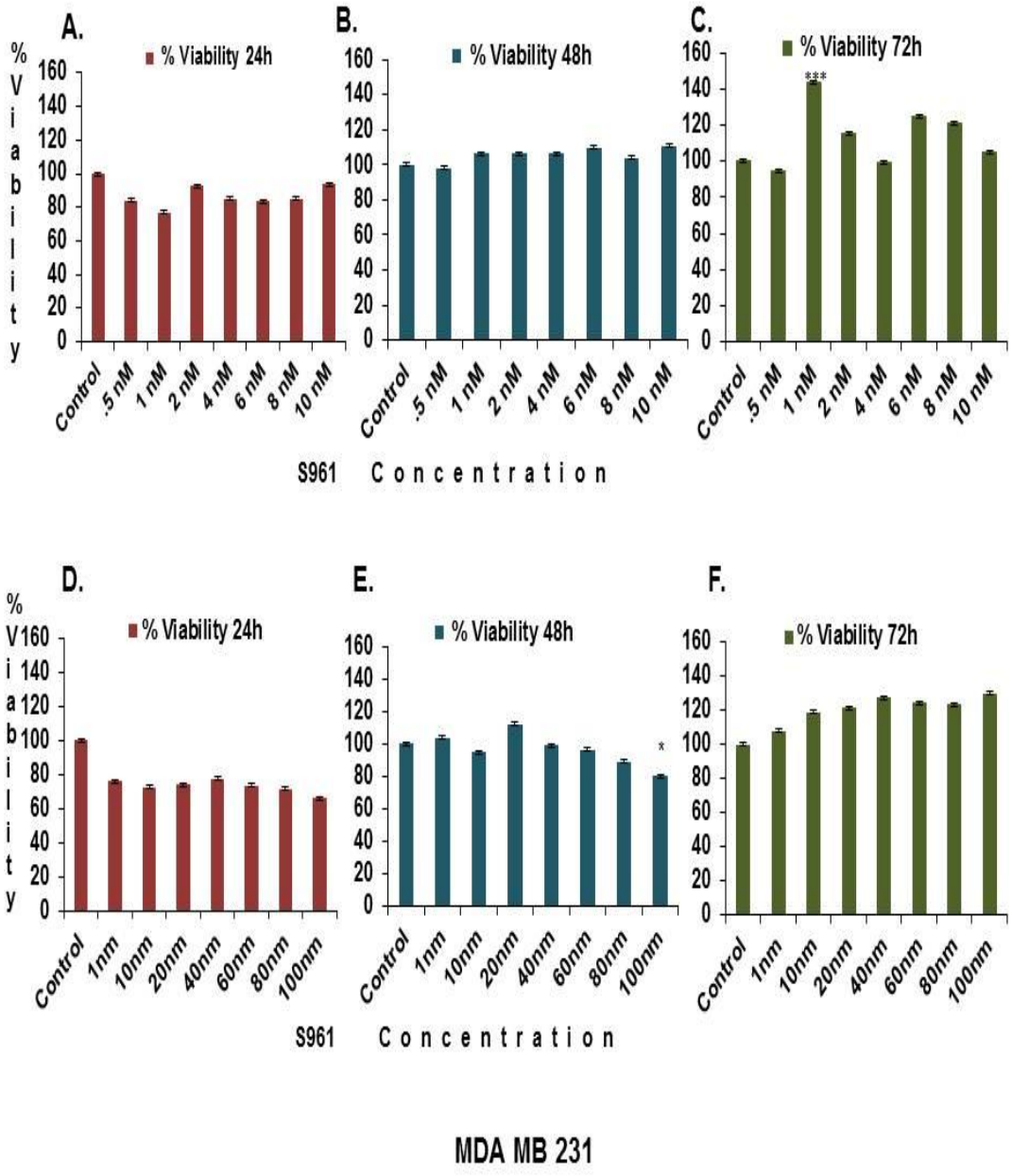

Figure 4.5.2: Effect of insulin receptor antagonist in presence of different insulin concentration on the breast cancer cell line MDA MB 231. *Figure A, B and C showed the effect of insulin receptor antagonist S961 (0.5nM-10nM) in combination with 100nM insulin concentration and figure D, E and F depicts effect of S961 (1nM-100nM) with 100nM insulin concentration for 24, 48 and 72h respectively in MDA MB 231 breast cancer cell lines. All the values shown in % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukeys test.*

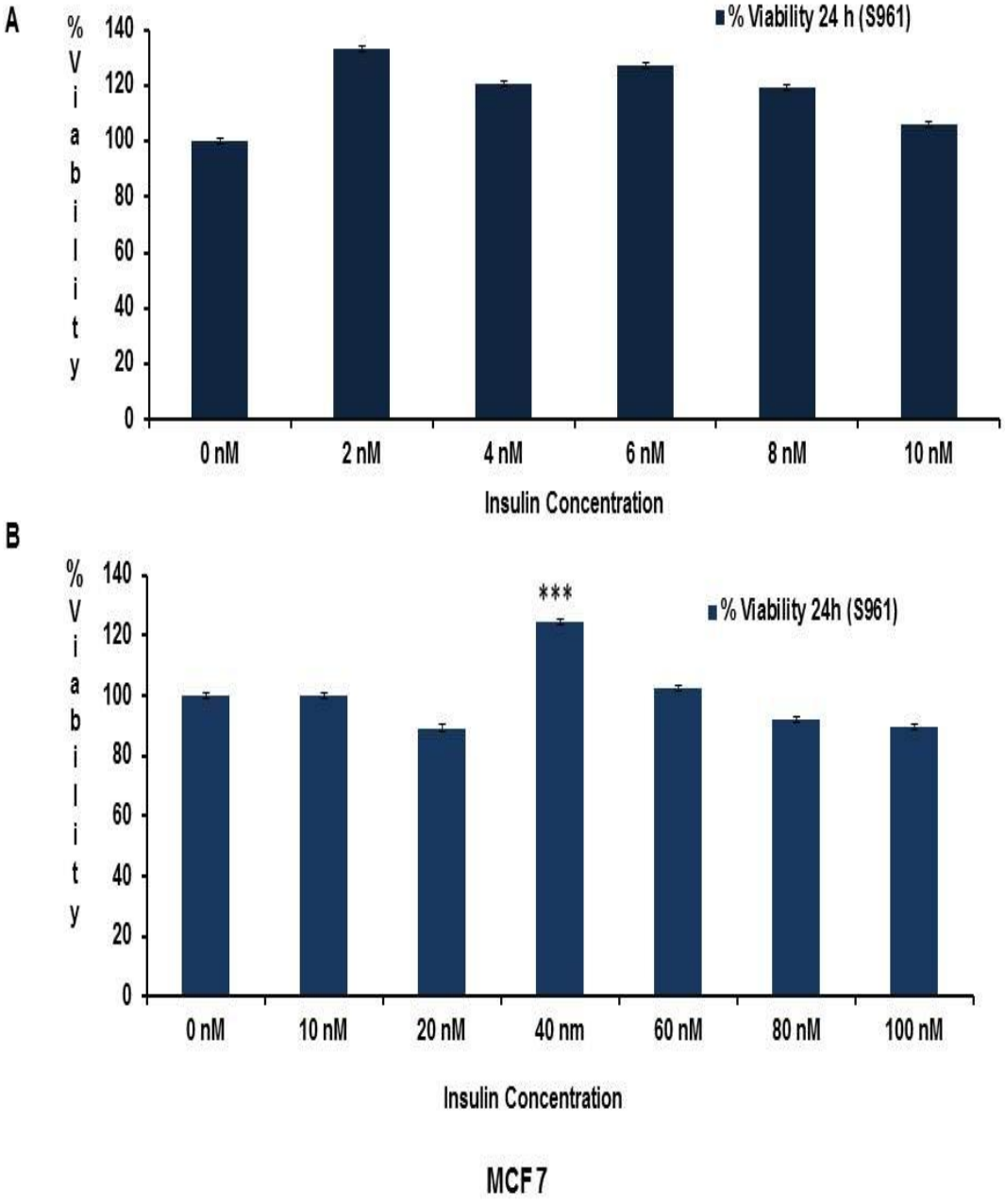

Figure 4.6: Effect of insulin receptor antagonist S961 in presence of different insulin concentration on the breast cancer cell line MCF 7. *Figure A showed effect of insulin receptor antagonist S961 (10nM) in combination with insulin (0 – 10nM) and figure B showed effect of S961 (10nM) in combination with insulin (0 – 100nM) for 24h. All the values shown in % viability ±SEM, data analyzed using t test: Paired two sample means and there was statistical significance difference between control and groups analyzed using ANOVA: One Way Analysis of Variance using Tukeys test.*

CHAPTER 5

DISCUSSION

Discussion

Cancer is long known as proliferative and metabolic disorder. Many factors which might not be involved in carcinogenesis but may act in tumor progression. For example any factor leading to increased metabolism may aid the cancer cell by providing extra energy. Diabetes is a complicated disorder which leads to defective metabolism of insulin and glucose. It has been associated with cancer as well. Since treatment of diabetes involve use of insulin, which in way is very potent mitogen. It became absolutely necessary to understand the complex relationship on how these mitogens may aid tumor development.

In the present study we have tried to test the proliferative effects of glucose and insulin on breast cancer cell lines. We also tried to see the efficiency of metformin drug used to treat diabetes as potent anticancer agent. For this study we used hormone dependent non metastatic fibro adenoma (MCF 7) and hormonal independent but highly metastatic breast cancer cell line MDA MB 231.

There are number of epidemiological studies showing the association between metabolic alterations and progression of cancer but there are less experimental studies available (Masur, et al., 2010). The present study indicates that metabolic alteration leads to proliferation of breast cancer cell lines. Glucose which is primary energy source for cell, also act as mitogen for the growth and proliferation of breast cancer cell lines, MCF 7 showed proliferation in dose and time dependent and MDA MB 231 showed ~3 fold proliferation at normal physiological glucose concentration, on further increase leads to saturation. As glucose is source of energy and involved in cell division. High glucose concentration induces increase in DNA synthesis, decrease in PKC ϐ II protein and mRNA levels causes glucose-stimulated proliferation in MCF 7 cells (Yamamoto *et al.*, 1999). High glucose level increases protein and mRNA level of GSK3ϐ in MDA MB 231 breast cancer cell lines (Vaira et al., 2012) and down regulate expression of p27 in MDA MB 231 breast cancer cell lines (Eto, 2011). High level of glucose promote proliferation and motility rate in tumor cells by the activation of PI3K, PKCŬ and MLCK (Masur, et al., 2010). It increases proliferation by up regulation of cdk2 and cyclin D1 level in cell cycle (Okumura *et al.*, 2002).

This study showed that insulin stimulates cellular proliferation in both estrogen dependent (MCF 7) and independent (MDA MB 231) breast cancer cell lines in dose and time dependent, also found that insulin independently and in presence of normal physiological glucose concentration or hyperglycemic glucose concentration stimulate proliferation. MCF 7 showed proliferation in 24h and 48h and sensitive to wide range of insulin concentration whereas MDA MB 231 showed proliferation in all time period and sensitive to lower insulin concentration. In comparison to insulin with normal physiological glucose treatment, MCF 7 showed significant proliferation at insulin and hyperglycemic glucose combination whereas MDA MB 231 is not sensitive to insulin and hyperglycemic glucose concentration combination as compared to insulin and normal physiological glucose concentration. By comparing the insulin independent and in combination with glucose it was concluded that insulin showed its proliferative effect independent of glucose. Insulin stimulates increase in cell proliferation in dose dependent manner in MCF 7 breast cancer cell lines (Chappell, et al., 2001) as IR is overexpressed (2-6 fold) in cancerous cells (Godsland, 2010). MCF 7 shows significant proliferation with hyperglycemic glucose concentration in combination with 100ng/ml insulin (Masur, et al., 2010). Insulin promotes protein synthesis by activating the mammalian target of rapamycin (mTOR) via phosphorylation of tuberous sclerosis complex 2 protein (Gonzalez-Angulo & Meric-Bernstam, 2010). MDA MB 231 cells are not responsive to higher insulin concentration such as 100nM due to reduced tyrosine kinase activity of insulin receptor (Costantino *et al.*, 1993). Plasma cell membrane glycoprotein-1 (PC-1) inhibits insulin receptor (IR) tyrosine kinase activity (Kumakura *et al.*, 1998; Maddux & Goldfine, 2000). Over expression of PC-1 inhibit insulin receptor tyrosine kinase activity in MDA MB 231 breast cancer cell line (Belfiore *et al.*, 1996) and in MCF 7 breast cancer cell line (Maddux & Goldfine, 2000).

It is observed that metformin, an antidiabetic drug, significantly inhibit the cell proliferation of MCF7 an MDA MB 231 in dose and time dependent manner, as it act through AMPK pathway and inhibiting the protein synthesis and result in inhibition of cell division. It shows its antiproliferative effect only at higher concentration as at lower concentration its effect was suppressed by hyperglycemic glucose concentration. Earlier studies suggests, it inhibit growth and proliferation in both estrogen dependent and independent breast cancer cell lines (Phoenix*, et al.*, 2009; Zakikhani*, et al.*, 2006), reduces colony formation, induces cell cycle arrest at G_1 check point (Alimova, et al.,

2009) also as recently reported induces apoptosis by arresting the cells in G_1 phase (Malki & Youssef, 2011). But some study shows that MCF 7 is sensitive to the growth arrest to metformin but MDA MB 231 is not sensitive as MDA MB 231 expresses lower level of p27Kip1 or p21Cip1 as compared to the MCF 7 breast cancer cell lines (Zhuang & Miskimins, 2008). Metformin inhibit growth in both MCF 7 and MDA MB 231 cell but more effective in MCF 7 as compared to the MDA MB 231 (Phoenix, et al., 2009). In MCF 7 cells metformin act as growth inhibitor rather than insulin sensitizer and also shows significant reduction in tumor growth and proliferation in MDA MB 231 as compared to other hormonal independent breast cancer cells (Gonzalez-Angulo & Meric-Bernstam, 2010). This study also suggested that metformin in supplement with glucose and insulin showed more inhibitory effect in breast cancer cell lines (MCF 7 and MDA MB 231). So, these results suggest that metformin is not only a treatment remedy for type 2 diabetic patients but also drug for diabetic breast cancer patients.

It is observed that the novel insulin receptor antagonist S961 did not show any inhibitory action in MCF 7 and MDA MB 231 breast cancer cell lines. There is no study on S961 on breast cancer cell lines. As in earlier studies it prevents insulin induced cell proliferation in PC-3 cell lines (Vikram and Jena 2010). Probably due to sensitiveness of the cell lines to the tested doses of insulin a much vide scope is still left unproven. It is expected that the S961 should be effective at much lower dose, may be at pico molar concentration of insulin. This will open up new frontier in establishing the relationship between insulin and insulin receptor antagonist S961.

These studies strongly confirm and support the proliferative effect of glucose and insulin on breast cancer cell lines in addition to their role in cell metabolism. And also support the inhibitory effect of metformin in both estrogen dependent and independent breast cancer cell line. There are also some reports which suggested that MDA MB 231 which is estrogen independent breast cancer cell line is not sensitive to metformin. But this study confirms and support to the other studies which shows MDA MB 231 also responsive to metformin.

SUMMARY

Summary

Breast cancer cell lines MCF 7 and MDA MB 231 showed proliferative effect when cultured under glucose. Hyperglycemic conditions accelerate cell proliferation when compared with physiological glucose concentrations and addition of insulin further enhances proliferation of these cell lines. Proliferation of these cell lines not only depends on the concentration but is also time dependent. Growth of these breast cancer cells are inhibited by antidiabetic drug metformin, which is used in the treatment of diabetes. Data accumulated from this study clearly indicate the role of glucose and insulin individually as well as in combination stimulate cellular proliferation in breast cancer cells and also suggests the role of antidiabetic drug metformin in growth inhibition of breast cancer cell lines (MCF7 and MDA MB 231) proliferation. Insulin receptor antagonist S961 did not show any inhibitory response in the MCF 7 and MDA MB 231 breast cancer cell lines probably due to high sensitivity of these cell lines and blocking effect of higher concentration of insulin.

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