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

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AUTHOR'S CONTRIBUTION

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

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

Keywords: Hyperinsulinemia, mitogen, PC-3 cell proliferation, insulin, MMPs activity.

1. INTRODUCTION

Cancer is one of the leading diseases responsible for high mortality rates worldwide. It is a multi-factorial group of diseases involving uncontrolled growth of cells and resulting in formation of tumors, malignant tumor can metastasize into surrounding tissues as well as in distant organs [1]. All cancer arises due to the change in DNA sequence of cancer cell genome. It involves over hundred distinct diseases with variety of risk factors and different epidemiology [1]. Cancer is same like an evolutionary process in which cells acquire mutations randomly and are selected based on the survivability. Cells acquainted with deleterious mutations are eliminated, while occasionally cells acquire advantageous mutations that allow uncontrolled proliferation and metastasis. Cancer cells have the trait to proliferate indefinitely and it is achieved through the release of mitogenic signals by themselves or inducing surrounding cells to release mitogenic signals also termed as paracrine signaling.

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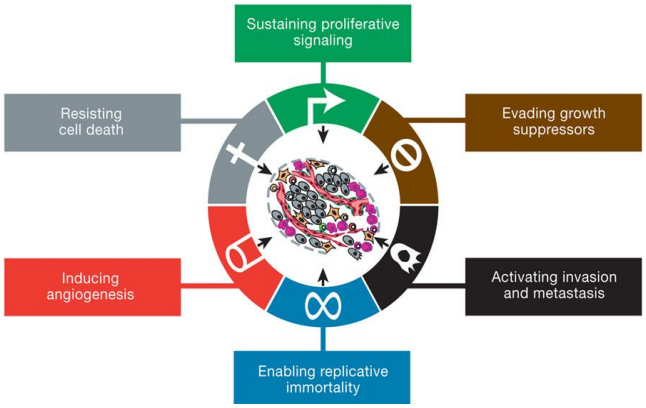

Fig. 1. Major hallmarks of cancer [2]

Prostate cancer is the major non cutaneous cancer diagnosed in the males in United States [3]. Prostate gland is found in men and is located in front of the rectum under the bladder. Prostate contains many small glands which make about twenty percent of the fluid constituting semen. Prostate cancer tends to develop in men over the age of fifty and this is one of the most prevalent types of cancer in men. Prostate cancer starts in the prostate cells and can invade and damage normal tissues. Many factors, including genetics and diet have been found to be associated in the development of prostate cancer. Prostate cancer incidence increased and mortality has decreased due to the early detection and introduction of blood based prostate specific antigen test [4]. Hyperinsulinemia has also been found to be associated with higher risk of prostate cancer [5, 6]. The presence of prostate cancer may be indicated by symptoms, physical examination and prostate-specific antigen (PSA), or biopsy. Common treatments for prostate cancer includes external beam therapy, interstitial radiation therapy, surgery to remove gland and androgen deprivation therapy [5]. Men with prostate cancer and increased body mass index tend to have lower PSA level as compared to thinner men with similar cancers. So this can delay prostate biopsy recommendation in obese persons and results increased risk of higher grade cancer [7]. Prostate cancer cell lines are used to investigate the mechanism involved in the progression of prostate cancer. LNCaP, PC-3 and DU-145 are most commonly used prostate cancer cell lines.

1.1 Diabetes and Cancer

Cancer is characterized by uncontrolled cell division and diabetes is a metabolic disorder characterized by hyperglycaemia. Both the diseases involve external as well as internal risk factors. Type-1 diabetes is characterised by hyperglycaemia, endogenous deficiency of insulin secretion and requires administration of exogenous insulin [8]. Type 2 diabetes shows hyperinsulinemia, hyperglycaemia and hyperlipidemia for prolonged time [9]. In type-2 diabetes exogenous insulin is required only when β cell function fails. Diabetes is a complex disease in which different factors interact to alter insulin action, secretion and resulting in hyperglycaemia [10]. Interaction between Prostate cancer and diabetes can be explained on the basis of hyperinsulinemia and hyperglycaemia conditions that may lead to increase in tumor proliferation and metastasis [11]. Insulin acts as a growth factor for prostate epithelial cells. Beside insulin, insulin like growth factor IGF-1 and IGF binding protein-3 have also been found to be associated with prostate growth [11]. Growth promoting effect by hyperinsulinemia is mediated by the activation of insulin receptor (IR), insulin like growth factor receptor (IGF-IR) and phosphoinositide 3 kinase (PI3K) pathway [9]. Insulin receptor expression increases in breast cancer tissue as compared to normal breast tissue. Insulin pre-eminent role is metabolic but it also has mitogenic effects in the malignant cells at receptor and post receptor level. Hyperglycaemia, oxidative stress and obesity results in increased risk of cancer in diabetes [8]. Cancer is associated with higher mortality in diabetic patients as compared to non-diabetic. Insulin analogues are used in the treatment of diabetes mellitus [12]. Insulin B10ASP was the first insulin analogue to be developed with single amino acid substitution. It shows tenfold increase in mitogenicity as compared to human insulin. Alteration in the structure of insulin increases its mitogenic effects as demonstrated by enhanced DNA synthesis and cell division. This mitogenic effect is due to the prolonged binding to the insulin receptor and cross reactivity with IGF-1 receptor [13]. Long-acting insulin analogue glargine, detemir and short acting analogues lispro, as part exhibits activity like IGF-1 in the cultured cancer cells. These insulin analogues exhibit *in vitro* proliferative and antiapoptotic activities in various

cancer cell lines as compared to insulin [12]. Molecular mechanism linking diabetes and cancer are multi-factorial and requires extensive and better designed study.

2. MATERIALS AND METHODS

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

2.1 Cell Culture Treatments

Prostate cancer cell line PC-3 was obtained from National Center for Cell Sciences (NCCS, Pune, Maharastra, India). Cell lines were cultured in DMEM (HiMedia/Gibco, invitrogen) media supplemented with fetal bovine serum (FBS) (Gibco, invitrogen) and streptomycin and Penicillin antibiotics (HiMedia, India / Gibco, invitrogen). Cells were cultured at 37^oC with 95% humidity and $5\%CO_2$. Cell lines were maintained and subculture according to their doubling times and incubated in CO_2 incubator (Eppendroff, UK). For treatments 5000 cells per well were seeded in 96 well micro titer plate (Tarson) and 200000 cells in 6 well plate.

2.2 Treatments Preparations

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

2.3 MTT Assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution was prepared by dissolving in the PBS at 0.5mg/ml concentration and then 100ul per well was added to cell culture micro titer plate. Incubated for 4hours at 37^0C, formazan complex formed by active cells is solubilized with DMSO (200ul per well) and absorbance measured at 570 nm [14] in Microplate reader (Biorad USA). Serum starvation was given for 12 hours. After treatment period MTT assay was done to check proliferation in PC-3 cells.

2.4 ROS Assay

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

2.4.1 Fluorescence intensity measurement

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

2.4.2 Spectrophotometric analysis

For spectrophotometric analysis, equal numbers of cells were seeded, after mentioned treatment periods; cells were taken into solution using trypsin and washed with 1X PBS. Staining was done by incubating the cells with DCFDA dye in 1X PBS on ice for 20 minutes. Cells were than washed three times with 1X cold PBS and OD was taken in UV-Visible Spectrophotometer (Shimadzu, Japan. Model: UV-2450). The measurement parameters for DCFDA were 488 nm excitation and 520 nm emission [15].

2.5 RT PCR

PC-3 cells were harvested from cell culture flask at 80% confluence by using trypsin. After cell counting in the neubauer chamber, cells were seeded in 6 well plate (200000 cells per well). Serum starvation of 12 hours was given and then four different insulin treatments 50nM, 500nM, 1000nM and 2000nM were given for 24 hours. PC-3 cells were trypsinised from 6 well plates and centrifuged to collect a pellet of cells. Cells were processed as per the manufacturer's instructional protocol for RNA isolation (Genei India). RNA was quantified using Nano Drop spectrophotometer to check quality and quantity of RNA. cDNA synthesis was performed by using cDNA synthesis kit (Genei, India) as shown in table (2). The RT - PCR conditions were 42°C for 1hour cDNA synthesis followed by 95°C for 5 minutes for mRNA denaturation.

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

Table 1. Treatment plans

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

Table 2. RT PCR components used for cDNA synthesis

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

Table 3. Components used in the PCR for SOD gene amplification

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

2.6 Primer Used

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

2.7 Gelatin Zymography

Zymography is SDS PAGE modified technique in which substrate is copolymerized with acrylamide gel to detect the activity of corresponding enzyme. In gelatin zymography gelatin is polymerized in acrylamide gel to detect the activity of MMPs. In this experiment MMPs level was checked in PC-3 cells after insulin treatment. PC-3 cells were harvested from cell culture flask at 80% confluence by using trypsin. After cell counting in the neubauer chamber, cells were seeded in 96 well plate (5000 cells per well). Serum starvation was given for 12 hours and followed by three different insulin treatments 10nM, 100nM, 1000nM and for 24 hours. Media was collected from each well and concentrated in the vacuum concentrator (Eppendorf Germany). 10% polyacrylamide gel was made in 0.1% SDS containing 1mg/ml gelatin. Culture supernatants diluted with 6:1 7X sample buffer (17.4%SDS, 7% sucrose and phenol red in 1M tris-Cl pH6.8). Run at 200V, 50mA for 45 minutes at 4°C. Six washes were given in 2.5% TritonX100 in water (5 minute each). Three washing were given in PBS (10 min for each washing). Gel was placed in PBS pH7.4 containing 0.9mM $CaCl_2$, MgCl2 and 0.001mM $ZnSO_4$. Incubated overnight at room temperature on dancing shaker (Tarsons). Gel was stained with Coomassie brilliant blue and then de-stained till clear bands were visible.

2.8 Data Analysis

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

Table 4. Primer sequences of SOD gene isoforms 1, 2

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

3. RESULTS

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

3.1 Insulin Induces PC-3 Cell Proliferation

In order to elucidate the exact role of serum, two sets of experiments were setup (1-with serum & 2-without serum) and cells were treated with different doses of insulin for 24, 48 and 72 hours. Cell proliferation was measured using % viability (transformed MTT absorbance). The results of insulin treatment in presence of serum revealed that PC-3 did not show any appreciable increase in the proliferation from initial 10nM to 100 nM concentrations, later it increased statistically (at 1000nM insulin concentration) up to 1.1-fold in 24 hrs. No change in proliferation was observed in 48 hrs with similar treatments. But 72 hrs treatments did change the rate of proliferation significantly. Cell proliferation increased significantly at 10nM, 100nM and 1000nM insulin doses, 1.16-, 1.20- and 1.25-fold increase was observed at 10, 100 and 1000nM respectively. On the other hand, in serum free culture set up, proliferation of Insulin treated PC-3 cells showed significant cell proliferation (1.2-fold) at 10nM treatment over control. 48 hours treatment also significantly enhanced the cell proliferation 1.13 and 1.11-fold at 100nM and 1000nM insulin doses respectively. Further increasing the time up to 72 hrs did not affect the proliferation rate over control.

4. DISCUSSION

Several epidemiological studies have revealed association between Diabetes and cancer [16]. Exogenous infusion of insulin can have effect on proliferation of cancer cells and also promoting metastasis. Hyperglycaemia hyperinsulinemia and obesity are important factors that correlate diabetes with cancer. All these conditions influence cell proliferation. Approximately 8 to 18% people with malignancies also have diabetes [17]. This is a well-known fact that insulin act as mitogen and increase cell proliferation. Present studies aim to investigate role of insulin in prostate cancer cell proliferation, ROS production and Superoxide dismutase expression. Recent study showed that Insulin treatment increases PC-3 cells growth in a dose dependent manner. Also, insulin antagonist S961 inhibits growth of PC-3 cells, which confirms that insulin acts as mitogen and has direct effect on proliferation [18]. Insulin analogues show mitogenic and antiapoptotic effect, although insulin analogues show more pronounced effect on growth of various cell lines as compared to insulin [12]. Initially, the PC-3 cells were tested with and without serum and insulin treatment. Initial experiments depict that insulin concentration at 10nM and 100 nM were not effective. PC-3 responded in same fashion with or without FBS. But without FBS cells fail to show any growth response with increase in the time. Cells grown with FBS were efficient in showing significant growth even in 72 hrs treatment. But when tested with broad range of insulin doses, PC-3 showed appreciable growth response even from 10 nM, which was significant over control. Since FBS contain essential growth factors that contribute to the survival of cells and hence can influence cell viability in 72 hrs treatment without FBS. Broad range of insulin dose was tested on PC-3 cells at 48 hrs time interval with FBS. A significant increase was recorded right from 10 nM (1.5-fold) to 2000nM (3 folds). It is quite evident from these observations that insulin is promoting PC-3 cells growth to a significant level. Oxidative stress results due to imbalance between steady state levels of intracellular pro-oxidants and antioxidants [19]. Increased level of reactive oxygen species and metabolic defects is recognised as the most common feature in different cancers. Superoxide anion is the primary oxygen radical that is generated in the different biological processes. It is further metabolized into hydrogen peroxide (H2O2) and hydroxyl radical (HO•) by plethora of antioxidant system at cellular level. Hydroxyl radicals the most reactive form of ROS that causes oxidative damage to DNA and proteins in the cell. Low level of ROS promotes cell proliferation by activation of growth promoting signaling pathways in normal cells. Chronic oxidative stress plays a vital role in prostate cancer initiation and progression. Abnormally high level of ROS in cells leads to a state of oxidative stress that is linked with carcinogenesis and cancer progression [15]. Increased lipid peroxidation and decreased efficiency of antioxidant system leads to production of epoxides. These moieties can covalently combine to DNA, RNA and protein which results in mutagenicity, cytotoxicity and carcinogenicity [20]. This study was carried out to know the effect of higher insulin doses on the level of ROS. PC-3 cells were treated with different insulin doses up to 24 hours. Intensity calculation shows that ROS production increased at higher insulin doses. In terms of fluorescent intensity of PC-3 cells 1.76-, 2.18-, 2.36- and 2.75-fold increase was observed which confirms increase in ROS level. Spectrophotometric analysis of Insulin treated and DCFDA stained PC-3

cells showed 2.22-, 2.54-, 2.39- and 2.68-fold increase in ROS levels with increasing insulin concentration (50-2000nM). Previous studies pertaining to oxidative stress has revealed that cancer cells show higher ROS level as compared to normal cells. ROS generation in these cancer cells are related with NAD(P)H oxidase (Nox) systems and with mitochondria. Cross talk between ROS generation and tumorigenic potential shows that ROS can modulate tumor growth. Oxidative stress contributes to increase in aggressive and invasive behavior in PC-3 cells [21]. So, insulin not only increases proliferation but may contribute in aggressive prostate cancer due to increase in ROS level. DNA damage and modification in protein folding process caused by oxidative stress is other phenomenon which is related with carcinogenesis. Superoxide dismutase is an antioxidant enzyme which clears ROS from cells. Superoxide generated in intracellular processes is dismutated into hydrogen peroxide. SOD plays an important role in saving aerobic life, as ROS is continuously generated in the biological processes. But ROS level is kept low by cooperative activity of antioxidant system [22]. SOD over expression leads to inhibition of tumor growth and decreased tumor aggressive nature [23]. RNA was isolated from treated and untreated cells. After cDNA synthesis PCR was carried out with SOD specific primers. Densitometric analysis of SOD1 reveals that insulin is promoting SOD1 expression (3.34 folds) at initial dose of 50 nM. But with further increase in dose up to 2000 nM, decrease in the expression was observed. Further increase in insulin level may be causing oxidative imbalance. Densitometric analysis of SOD2 has also shown same trend increase in expression. At 50nM insulin treatment 1.07-fold increases was observed in comparison to control. Interestingly, further increase in treatment decreased the SOD2 expression level even lower to the control. Spectrophotometric analysis and fluorescent imaging revealed that ROS level increases with higher insulin doses, this increase in ROS level can be attributed to lower level of SOD gene expression. Free radicals can't be removed efficiently due to lower expression of SOD gene and it may lead to oxidative stress. So, insulin may increase tumor's aggressive and invasive nature by modulating SOD expression and hence ROS level in cancer cells. Other studies regarding SOD expression in cancer cells have shown that different isoforms of SOD are expressed at varying levels depending upon the stage of cancer. Therapies involving modulation of SOD expression should be based on type and stage of cancer [24]. MMPs are family of proteases secreted by cells or associated with cell membrane. MMPs degrades extracellular matrix not only in the normal conditions but also has a role in the pathological conditions such as metastasis in cancer. Extracellular matrix (ECM) provides a structural framework to support cells and also mediates cell-cell and ECM-cell interaction. ECM is composed of proteoglycans, elastin and collagen. MMPs are synthesized in the latent form and converted later on in to active form (Ii et al., 2006). They can control microenvironment around cell through extracellular proteolysis. MMPs are divided into eight groups of which five are secreted while three are membrane associated [25, 26, 27]. Our result shows that higher insulin doses can increase MMPS expression. MMPs expression increased with increasing dose of insulin. Previous studies have shown that hyperglycaemia and higher insulin doses can increase migratory activity in various cell lines [16]. On the basis of these results, we can say that insulin affects tumor growth and invasion by increasing MMP level and ROS level in cancer. All these factors can work in a synergistic way.

5. CONCLUSION

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

5.1 Dose Dependent Effect of Insulin on PC-3 cell Proliferation

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

5.2 Insulin Increases Free Radical Stress in PC 3 Cells

Since insulin can directly affect cellular metabolism in number of ways which might be reflected upon increased cell proliferation. Free radicals are closely related to cell metabolism, whose alteration may

change level of ROS inside a cell. Our hypothesis is that with increased cell metabolism, there will be hyper production of free radicals, which in-turn may lead to cell transformation (hyper-proliferation) through direct DNA damage or through dysregulation of various signaling pathways. We thus aim to investigate the status of free radicals in PC-3 cells with or without insulin treatment. PC-3 cells were treated with different insulin doses for 24 hours and stained with DCFDA dye for detection of ROS by fluorescent microscope followed by comparison of cell intensity (10 cells per treatment in triplicate). Fluorescent images for free radical levels measured by DCFDA (in green; Fig. 3) show a dose dependent increase.

We next wanted to compare the relative staining intensity from all the samples. The staining was done in triplicate and from each sample, 10 cells were used to measure intensity using Image-Pro software from Nikon (10 X 3 = 30 cells per time point). The plotted graph showed progressive increase in ROS at higher insulin doses. There was 1.76-, 2.18-, 2.36- and 2.75-fold increase in ROS staining intensity (at 50nM, 500nm, 1000nM and 2000 nM respectively; Fig. 4) as compared to control PC-3 cells.

We further processed the DCFDA stained cells for spectrophotometric analysis. Our result showed that ROS level increases with the higher dose of insulin. Increase in ROS level was observed 2.22, 2.54, 2.39 and 2.68-fold in 50nM, 500nm, 1000nM and 2000nM insulin treated cells over control (Fig. 5). Statistically increase was highly significant at 50nm, 500nM, 1000nM and 2000nM as compared to control. Optical density was transformed to fold intensity which was statistically significant over control and within treatments. Increase was significant at 50nm, 500nM and1000nM respectively.

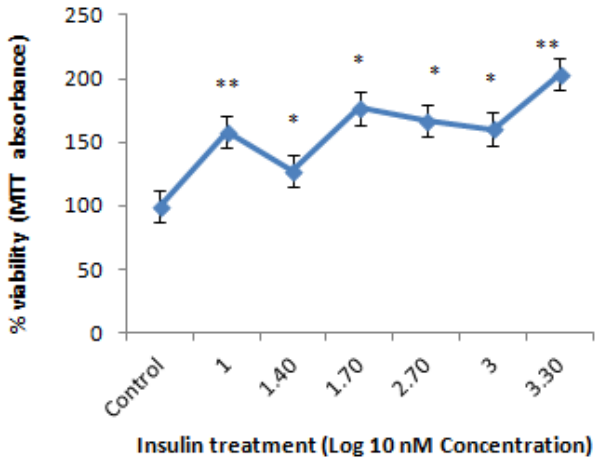

Fig. 2. PC-3 cell proliferation after 48 hours treatment of insulin (with FBS). Insulin doses used in the experiments were 10nM, 25nM, 50nM, 500nM, 1000nM and 2000nM

**, P≤0.05 **, P≤0.01*

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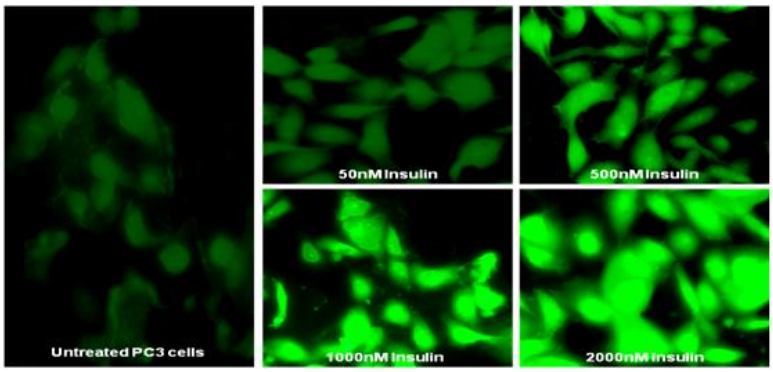

Fig. 3. Fluorescent images of PC-3 cells treated with different insulin doses for 24 hours and stained with DCFDA dye for detection of ROS. Images were captured in Olympus fluorescent microscope (FSX 100) at 20X

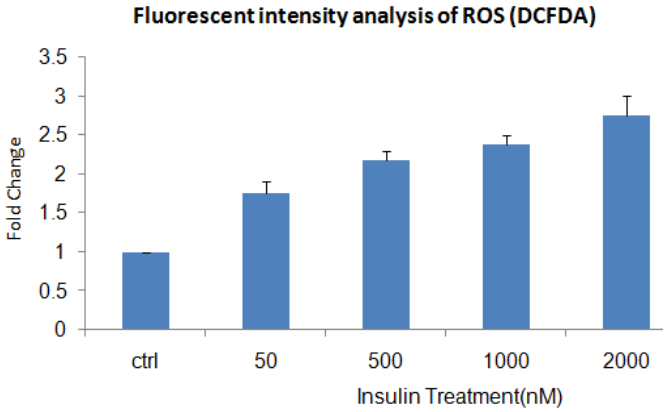

Fig. 4. Graphical representation of fluorescent intensity analysis of PC-3 cells for ROS detection after insulin treatment

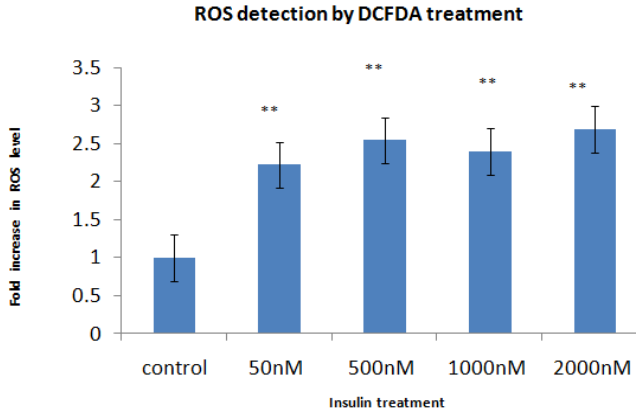

Fig. 5. Spectrophotometric analysis of Insulin treated PC-3 cells stained with DCFDA for ROS detection
***, P≤.01*

5.3 Superoxide Dismutase Expression with Insulin Treatment

Till now our results show that insulin treatment showed an increase in free radical levels. Hyper ROS can be harmful in number of ways like causing direct DNA damage, formation of protein aggregates as well as dysregulation of cell signaling pathways, so it's scavenging becomes of utmost importance. There are number of enzymes involved in scavenging like superoxide dismutase family. So, our next goal was to investigate the effect of ROS increase on expression of SOD family members (SOD1 & 2). Insulin treatment doses were selected on the basis preliminary study of proliferation mediated at these concentrations. Insulin treatments 50nM 500nM 1000nM and 2000nM were given to PC-3 cells for 24 hours' time period to check the superoxide dismutase expression. RNA isolated from treated and untreated cells were subjected to two steps RT PCR (cDNA synthesis followed by PCR with SOD specific primers). The PCR product was viewed in 1.5 % agarose gel. Gel image was taken in geldoc (Biorad) and at the same time densitometric analysis was also carried out to check the band density. SOD1 PCR product was of 215bp (base pairs), while SOD2 PCR product was 165bp in length. In general, SOD1 expression increased at 50nM insulin treatment but at higher concentrations it decreased appreciably (Fig. 6 A). Further increase in insulin level may be causing oxidative imbalance. Densitometric analysis of SOD1 RT PCR revealed that at 50 nM and 2000 nM insulin treatment SOD1 expression increased 3.34 and 2.65 folds respectively as compared to control (Fig 6 B). Same trend was observed in SOD2 expression after insulin treatment. At 50nM insulin treatment SOD2 expression increased 1.07-fold, which later on decreased with higher insulin doses (Fig. 6 C).

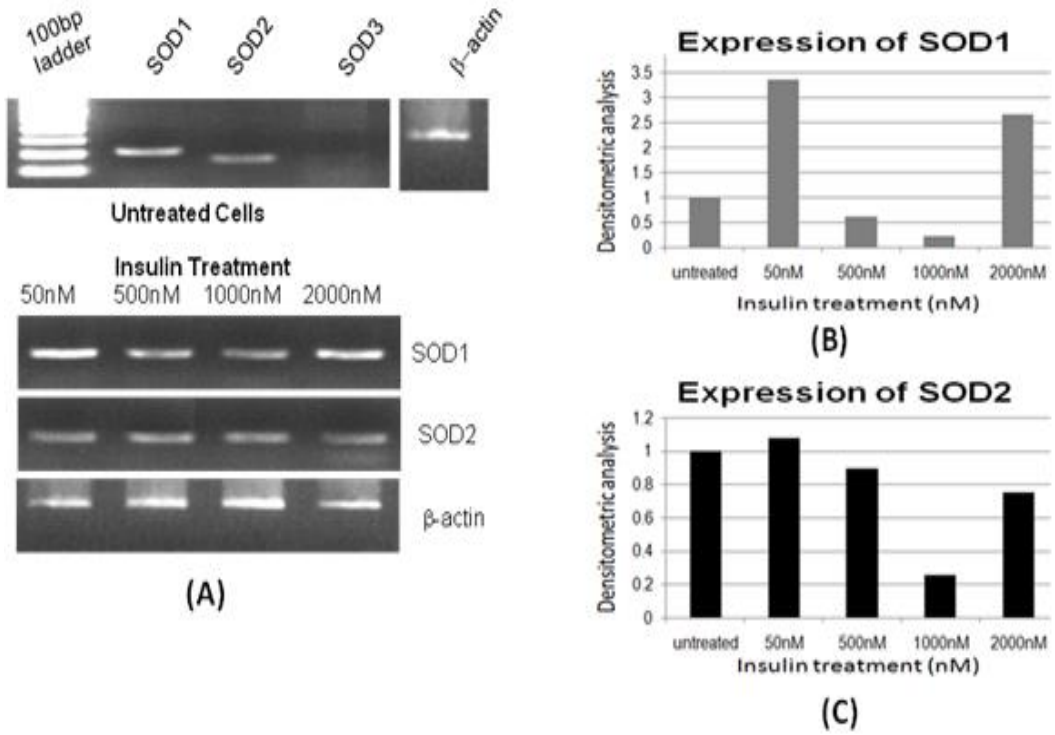

Fig. 6. Superoxide dismutase expression in response to Insulin (A) SOD 1, 2 and β actin, expression in the control (Untreated PC-3 cells) and treated cells. (B) Densitometric analysis of SOD1 expression (C) Densitometric analysis of SOD2 expression after insulin treatment at 50, 500, 1000 and 2000nM concentration after 24 hours

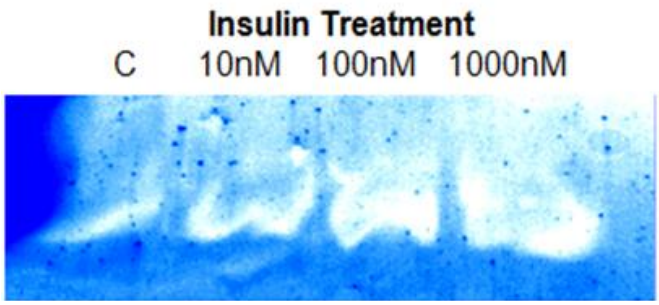

Fig. 7. Zymography gel showing activities of MMPS at different concentrations of insulin in PC-3 cells

5.4 MMP Expression after Insulin Treatment

Since free radical can cause increased cell proliferation as well as dysregulated movement of the cells, we wanted to investigate whether insulin treatment can cause the same effect on PC-3 cells or not. MMPs are Proteases which degrade extracellular matrix and hence increase in the expression of MMPs can enhance metastasis in cancer. As extracellular matrix degradation is a major step in metastasis. Zymography was done to know whether insulin treatment has any effect on the expression level of MMPs. PC-3 cells were treated with 10, 100 and 1000nM doses of insulin and media was collected and processed for the zymography assay. Clear bands in the zymography gel shows MMPs has degraded their substrate (Gelatin), which is copolymerized with the gel. It is evident from this experiment that band clarity increased with higher doses of insulin (Fig. 7). So, with the increase of insulin doses MMPs are secreted at higher levels and can have implications in metastasis.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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