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A Review on Effect of Anti-Diabetic Drug on Polymorphonuclear Leukocytes (PMNS) Adhesion and Apoptosis

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ABSTRACT

Diabetes mellitus is a metabolic and endocrinological disorder resulting from impaired insulin secretion, insulin action or both. It is characterized by increasing glucose levels in the body for a prolonged period. It increased the chances of infection by changing the immune system including innate and adaptive immune responses. Hyperglycemia or increased glucose concentration in blood neutrophil migration, phagocytosis and microbial killing, and apoptosis. Anti-diabetic drugs that revert glucose levels to normal thus possibly also modulate immune responses including neutrophil functions. In this study, we aim at evaluating the effect of anti-diabetic drugs on the neutrophil adhesion and apoptosis. Neutrophils were treated with anti-diabetic drugs (Metformin and Pioglitazone) for 30 min at RT and then treated with stimulant (fMLP). fMLP treated cell shows increase adhesion to extracellular matrix While no effect on adhesion was observed after Metformin and Pioglitazone treatment. In this study, we observed glucose up to 20mM concentration lead to an increase in adhesion, while severe hyperglycemia i.e. 40 mM glucose led to decrease in adhesion. In the next study, apoptosis analysis was performed in control and metformin treated cells that were stained with FITC-Annexin V and PI (propidium iodide) and were analyzed using flow-cytometer. We found that increased cell apoptosis and decrease cell survival after 18 hours fMLP treatment. Metformin treated cell shows protective effect and increased survival rate and decrease the cell apoptosis. Overall, metformin seems to protect neutrophil from apoptosis, while no effect is observed on adhesion.

INTRODUCTION

Diabetes mellitus is a metabolic disorder resulting from impaired insulin secretion, insulin action or both. It is characterized by the increased glucose levels in the body for a prolonged periods lead to chronic hyperglycemia (high level of plasma glucose) causing defects in carbohydrates, fats, and proteins metabolism. In facts, insulin action and insulin secretion regulates glucose homeostasis: a elevated level of glucose concentration activates insulin secretion that lowers the plasma glucose level [1, 2].

There are two types of diabetes mellitus-

1. Type 1 diabetes mellitus (Insulin dependent diabetes mellitus)
2. Type 2 diabetes mellitus (Non - insulin dependent diabetes mellitus)

Type 1 DM is chronic autoimmune disease related to immune-mediated destruction of insulin producing pancreatic β- cells leads to insulin deficiency. It comprises the 10% of all DM cases. In type 1 DM, glucose level is too high because pancreatic β-cells does not make hormone called insulin. Lack of insulin leads to uncontrolled breakdown of fat and high levels of free fatty acids in the plasma that decrease glucose metabolism in peripheral tissues such as skeletal muscle. Decrease peripheral tissues metabolism leads to high glucose levels. This is also known as insulin dependent diabetes Mellitus [3, 4].

Type 2 diabetes mellitus is considered as life style disorder that mainly depends on physical latency, inactive way of life, cigarette smoking and utilization of alcohol. In type 2 DM, pancreas produce insulin but fat, muscle and liver cells unable to respond insulin, also known as Insulin resistance. Body required insulin to help glucose enters cell but due to insulin resistance, too much glucose stay into the blood stream. This type of diabetes also known as 'Non-Insulin Dependent Diabetes Mellitus' [5, 6]. Inflammation is a defense mechanism of body immune response against microbial infections including viruses, bacteria, fungi and various parasites. Furthermore, immune system and inflammation play important role in recovery from tissue injury. It has been well reported that chronic inflammation can lead to tissue damage also. Meta-inflammation (low level of inflammation) is common in diabetic condition and shown to play important role in insulin resistance, obesity and diabetes. Obesity due to a lack of exercise is governed by a decrease in muscle mass, enhance insulin resistance. The alteration in dietary energy sources,especially he increase in fat intake, decreased starch intake, increased the consumption of simple sugars, and decreased dietary

fiber intake, contribute to obesity and cause impairment of glucose tolerance and increased the risk of type 2 diabetes[7].

Neutrophils are the first white blood cell which recognized the pathogen and reached to the site of inflammation. These cells were first discovered by the 'Paul Ehrlich' in 1879. The term polymorphonuclear leukocyte was given by 'Elie Metchnikoff' in 1893 [8]. PMNs eliminates the bacteria by multiple means such as oxygen dependent and oxygen-independent processes. Oxygen dependent bactericidal activity comprises the superoxide production by the enzyme known as NADPH oxidase. Neutrophils possess antibacterial proteins (Cathepsins, defensins, lactoferrin and lysozyme) [9].These antimicrobial proteins are secreted by neutrophil granules and delivery into the bacteria containing vacuoles (phagosome) that initiate pathogen killing, microbe removal and phagocytosis [10,11]. Neutrophils degranulation is the process in which neutrophils granules releases antimicrobial content or proteolytic enzyme found inside the cells. There are 3 types of granules and it is different by their contents - 1. Primary granules 2. Secondary granules 3. Tertiary granules

- Primary granules are also known as Azurophilic granules. The main role of primary granules is killing and degradation of engulfed microbes in the phagolysosome.
- Secondary granules, also called as Specific granules. It is most numerous type of granules.
- Tertiary granules, also known as Gelatinase B granules. Tertiary granules are something different from secondary granules because it has low content of antimicrobial substance [12].

Major drugs in use for treatment of T2DM-

There are various drugs which is used for the treatment of diabetes mellitus and lowers the blood glucose level in diabetic patient.

Table :- Anti-diabetic drugs [13**]**

S. No.	Category	Drugs	Mode of action
1.	Sulfonylurea	Gliclazide, Glimepiride, Glyburide	It inhibits the ATP-sensitive Potassium channel in the pancreatic beta cells results in stimulates insulin release by beta cells.
2.	Biguanides	Metformin, Phenformin	Metformin activates the AMPK that inhibits hepatic glucose production, improves insulin sensitivity and glucose uptake by muscle.
3.	Thiazolidinediones (TZD)	Pioglitazone, Rosiglitazone	Pioglitazone acts on the adipose tissue and muscle to stimulate the insulin sensitivity, to activates the glucose utilization via liver, and inhibits the glucose production.
4.	Alpha glucosidase inhibitors	Acarbose	It inhibits the alpha glucosidase enzyme that is involved in digestion of carbohydrates. Low level of sugar molecules is absorbed because carbohydrates are not lysis into glucose molecules and maintain the glucose level in the blood.
5.	Dipeptidyl-peptidase-4(DPP-4) Inhibitors	Linagliptine, Sitagliptine, Alogliptine	It stimulates the incretin level (Glucagon like peptide (GLP-1) that inhibits the glucagon secretion leads to increase insulin release and decrease glucose level.
6.	Glucagon-like peptide-1(GLP-1) agonist	Liraglutide, Dulaglide	Glucagon like peptide is associated with protective effects. It activates the incretin level that decreases the blood glucose level by activating the insulin release.
7.	Sodium glucose cotransporter 2 (SGLT2) inhibitors	Dapagliflozine Ertugliflozine	These antidiabetic drugs inhibit the renal glucose reabsorption that leads to lower glucose level.

In this study, we focused on Metformin and Pioglitazone, so these drugs characteristics, mode of action, and side effects are described in details here.

Metformin:-

Metformin, phenformin and buformin are biguanide family members. Metformin and other biguanides agent are obtained from **French Lilac or Goat's Rue** (Galega Officinalis). Metformin is safest oral hypoglycemic drug. It is used in the treatment of type 2 diabetes mellitus and most widely prescribed antidiabetic drugs [14]. Metformin inhibits hepatic gluconeogenesis and increase peripheral glucose uptake. It does not promote insulin release from pancreatic Beta cells. Metformin reduces weight gain, hyperglycaemia and hyperinsulinemia in those with type 2 daibetes mellitus. Metformin suppress the glucose production by liver and it also acts as an insulin sensitizer [15].

Mechanism of action:-

The major site of action of metformin is in the mitochondria. Metformin inhibits the activity of mitochondrial respiratory chain complex 1 by inducing AMPK activation that maintain the integrity of intestinal barrier. Activation of AMPK in liver shown to be mechanism by which metformin inhibits the LPS (lipopolysaccharide) levels in the liver. After distribution to the liver from the intestine, metformin inhibits the glucogenesis via four mechanisms -

1) Activation of hepatic AMPK via Liver kinase B1, that inhibits the mammalian target of rapamycin (mTOR) signaling.
2) Inhibition of Cyclic AMP (cAMP) production by blocking the adenylcyclase hich is induced by glucagon.
3) Inhibition of mitochondrial respiratory chain complex 1 to reduce ATP level and increase AMP/ATP ratio which induced AMPK [16].
4) Activation of AMPK inhibits hepatic glucose production, improves insulin sensitivity and glucose uptake by muscle.

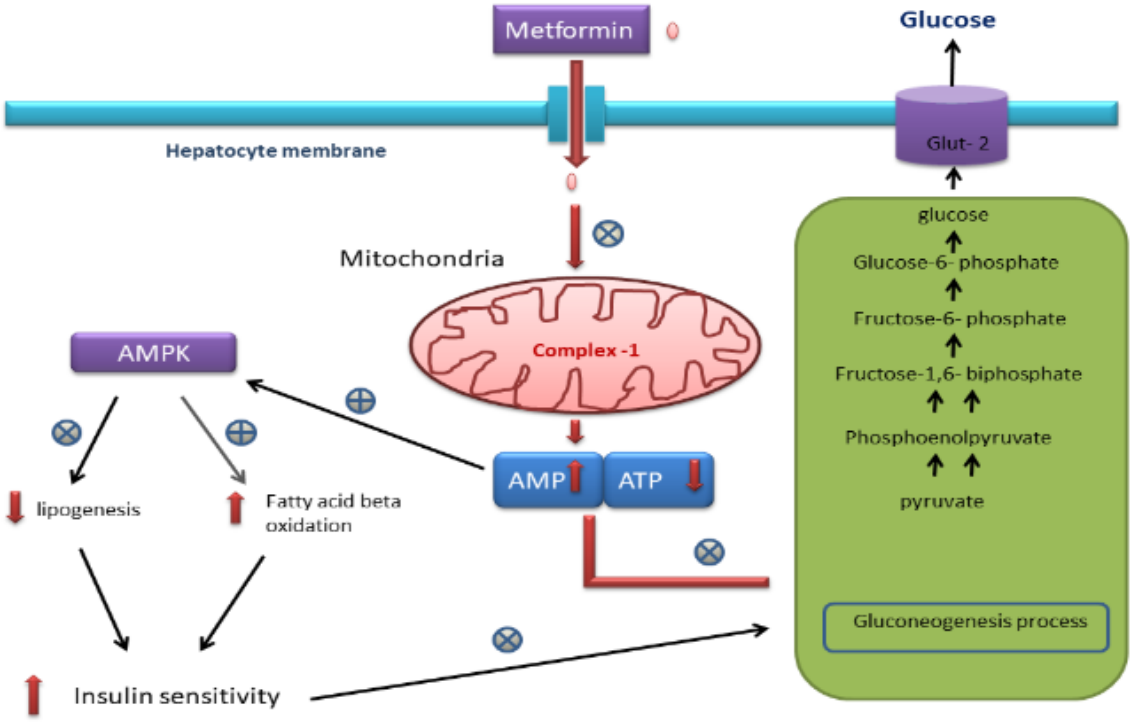

Figure 1-Mechanism of action of metformin

Pioglitazone -

Pioglitazone and rosiglitazone are belongs to the family of Thiazolidinediones. Pioglitazone is a oral antidiabetic drug used along with diet and exercise to maintain the high blood sugar in patients with type 2 DM.

Mechanism of Action :-

Pioglitazone binds to nuclear transcription factor PPAR-gamma (Peroxisome proliferator activated receptor gamma) in adipose tissue to promote the adipogenesis and fatty acid uptake. The location of PPAR-gamma is in CNS, macrophages, adipose tissue and β cells of pancreas. The content of PPAR-gamma is increased in skeletal muscle of diabetic patients. PPAR-gamma stimulates the weight gain by activating increased feeding. Pioglitazone acts on the adipose tissue and muscle to stimulates the insulin sensitivity and to activates the glucose utilization via liver and inhibits the glucose production [17].

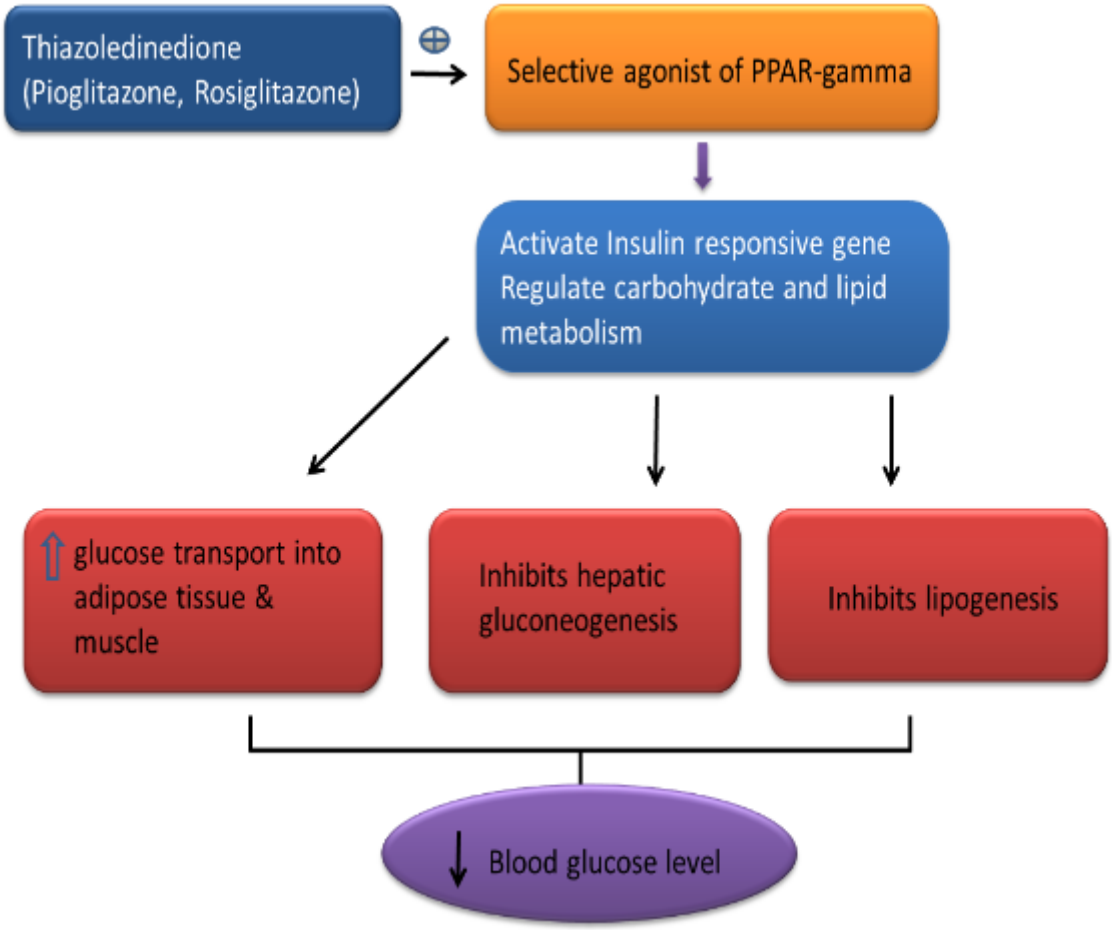

Figure 2- Mechanism of action of Pioglitazone

MATERIALS AND METHODS

Table No. 1: - List of chemical

Sr. No.	Chemical name	Source
1.	Fibrinogen	Sigma
2.	Collagen	Sigma
3.	Metformin	Sigma
4.	Pioglitazone	Sigma
5.	Bovine serum albumin	MP Biomedicals
6.	Glucose	Sigma
7.	Percoll	GE Healthcare
8.	Formaldehyde	Merck
9.	Ethanol	Merck
10.	Magnesium chloride	Sigma
11.	Calcium chloride	Sigma
12.	N-formyl-methionyl-leucyl-phenylalanine (fMLP)	Sigma
13.	Di ethyl ether	SRL
14.	Sodium chloride	Molychem
15.	Potassium chloride	Sigma
16.	Potassium phosphate mono basic (KH_2PO_4)	Sigma
17.	Di methyl sulfoxide	Fischer scientific
18.	Histopaque 1083	Sigma Aldrich
19.	Lipopolysaccharide	Sigma Aldrich
20.	DAPI(4,6-diamidino-2-phenylindole)	Ultra cruz
21.	Goat anti-rat Alexa fluor 488	BD Pharmingen
22.	Biotin Rat Anti-mouse antibody Integrin α [M chain] Mac1-α	BD Pharmingen
23.	Hepes sodium salt	Sigma Aldrich
24.	FITC Annexin V	BD Pharmingen
25.	APC-Cy7 Rat anti-mouse Ly-6G	BD Pharmingen
26.	Propidium iodide (PI)	BD Pharmingen
27.	Di ethyl ether	SRL
28.	Sodium phosphate dibasic dehydrate (Na_2HPO_4)	Sigma

List of Equipments

Sr. No.	Equipment	Source	Uses
1.	Microscope	Nikon ECLIPSE	It is used to viewing the object which are not view the naked eye. It uses light to passed through the sample to generate an image.
2.	Fluorescence Microscope	Leica	It is fluorescence microscope that uses fluorescence to developed the image with better resolution of fluorescence.
3.	Electronic balance	Gyan scientific	It is used to quickly and accurate measure the weight of the compound or substance.
4.	Incubator	Scientifics Biotech	It is used to maintain the humidity, temperature and provides the safe and contaminated free environment.
5.	Centrifuge	HERML ELABOR TECHNIK	It is used for the separation of liquids and fluid based on density.
6.	Flow cytometry	BD FACS Aria Fusion	It is used for the measurement of physical and chemical properties of cell or particle.

Reagents -

Metformin was dissolve in distilled water and used at concentration of 5mM (stock concentration 100mM). Pioglitazone was dissolved in DMSO (Dimethyl sulphoxide), used at concentration of 10µM (Stock concentration 10mM). fMLP was dissolved in DMSO and used at concentration of 10µM or 5µM (stock concentration 10mM).

Protocol 1- Neutrophils adhesion assay

Preparation of plate:-

- Adhesion assay was performed using multiwell plate.

- Multiwell plate was coated with fibrinogen (25 µg/ml) and acid soluble collagen (50ug/ml) and incubate for 2 hours at 37 degree Celsius. After 2 hour, plate washed with 1x HBSS.

- Blocking with 1% BSA for 20 min to remove non-specific binding.

- After blocking, wash with 1x HBSS for 2 times.

Adhesion Assay-

- Neutrophils suspension (30,000 cells/well) were added in collagen or fibrinogen coated plate.

- Incubate the plate with cells for 15 min at RT to settle the cells to the substrate.

- Add inhibitors Metformin (5mM) and pioglitazone (10µM).

- Incubate for 30 min at RT.

- After 30 min incubation, add 20 µl stimulant (fMLP 10µM) in each well and plate incubate for 10 min at 37 degree Celsius.

- Adherent cell was fixed by adding 20µl/well of 10% NBF (neutral buffer formalin) or 2% PFA (Paraformaldehyde) for 15 min at RT.

- At last, washing with 1x HBSS for 2 time to remove the non-adhering cell.

- The number of adhering cells on plate were examined under the microscope [18].

Protocol 2:- Apoptosis Detection by Flow Cytometry

- Apoptosis assay was performed by the flow cytometry analysis of control and metformin treated cells that were stained with FITC-Annexin V and propidium iodide.
- Neutrophils were suspended at the density of 1×10^6 cell / ml in RPMI-1640 supplemented with 10 % fetal bovine serum.
- Cell has cultured in 24-well plates and treat with different apoptotic inducer for 2 and 18-20 hours at 37 degree Celsius in a 5% CO2 incubator.
- After 2 hours incubation, take 300 µl cell from 24 well plate and then centrifuge for 5 min at 1500 rpm.

- After that, discard the supernatant and pellet in 100 µl annexin V binding buffer.
- Then, Cells were stained with Annexin V- FITC (1µl) and Propidium iodide (.1µl) and incubate for 20 min at RT in dark condition.
- After 20 min, add 500 µl of HBSS-BSA and centrifuge for 5 min at 1500 rpm.
- Then discard the supernatant and resuspend the pellet with 200 µl HBSS –BSA.
- After that, samples were immediately analyzed via flow cytometry.
- Annexin V staining was detected as green fluorescence and PI as red fluorescence [19].

RESULTS

Standardization of fMLP induced PMNs adhesion Assay -

Ifirst studied the effect of fMLP on neutrophils adhesion, as a known stimulator. Coating of extracellular matrix such as fibrinogen induced the significant adherence of neutrophil up to 50%. Neutrophils were incubated with fMLP (10µM) and adhesion was monitored as per protocol described in method section. Light microscopy in figure 2 showed that fMLP treated cells shows alter the morphological structure and induced the adherent of cell to extracellular matrix as compared to control. Statistically significant effect was observed in comparison to control.

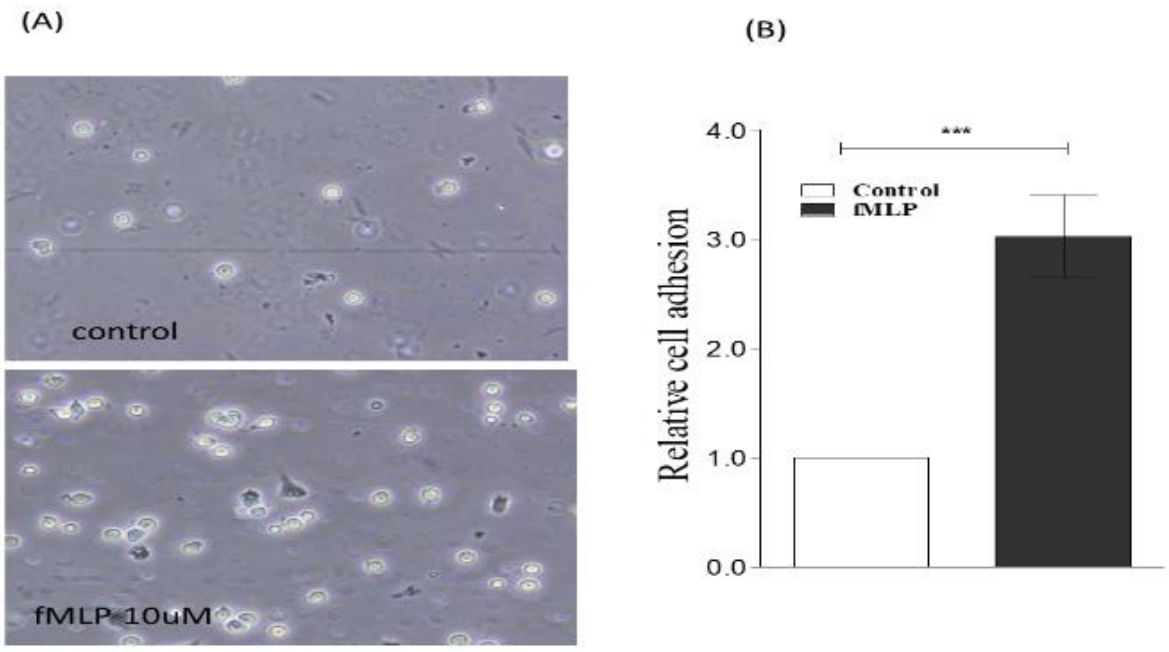

Figure 3-Standardization of neutrophils adhesion to extracellular matrix (Fibrinogen)

(A) Representative image of neutrophil treated with fMLP (10µM) **(B)** Neutrophil were incubated with fMLP (10µM). fMLP (10µM) treated cell shows significant effect as compare to control. Data is represented as the mean ± SD (***P< 0.001).

Effect of hyperglycemia on neutrophil functions: -

Effects of different glucose concentrations on neutrophils adhesion followed by fMLP:-

Neutrophils isolated from mice were cultured in presence and absence of glucose concentration followed by stimulation with fMLP(10µM). PMNs were incubated with different glucose concentration (5mM, 10mM and 20mM and 40mM) for 30 minutes in the presence of fMLP. PMNs adhesion increased in concentration dependent manner under different glucose levels (5mM, 10mM and 20mM). I observed glucose upto 20mM concentration which lead to an increase in adhesion, while 40 mM glucose led to decrease in adhesion but do not show any significant effects.

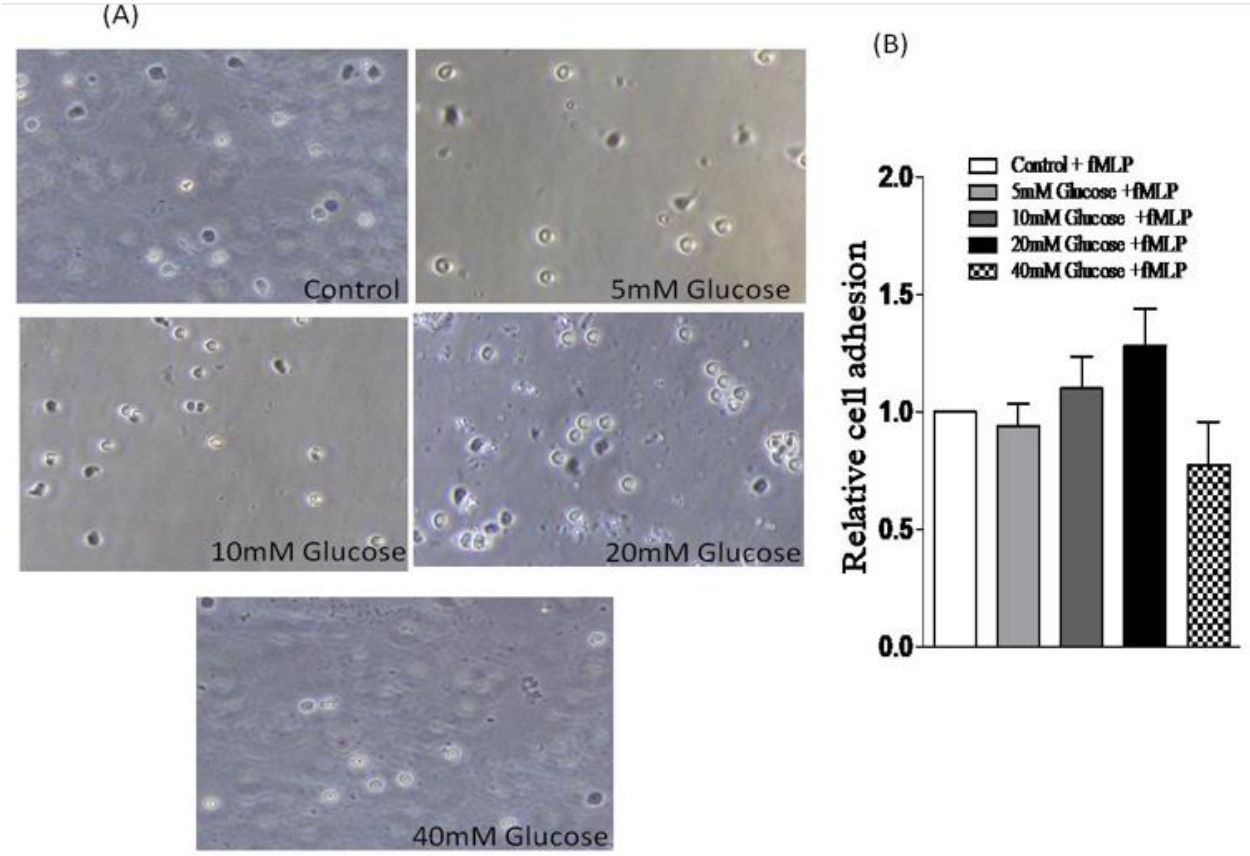

Figure 4- Neutrophils treated with increased glucose concentrate dependent manner followed by fMLP. (A)

Representative image of neutrophil treated with different glucose concentration (5mM, 10mM, 20mM and 40mM) followed by fMLP for 30 minutes **(B)** Bar graph showing relative cell adhesion in response to glucose treatment in the presence of fMLP (10uM).

Effect of anti-diabetic drugs on neutrophil functions

Effect of metformin on Neutrophil adhesion in the presence of different glucose concentrations-

To identified the impact of metformin (5mM) on neutrophil adhesion. Neutrophil were incubated with different glucose concentration (5mM, 10mM and 20mM and 40mM) in the presence of metformin (5mM) for 30 minutes. 20mM glucose show increased cell adhesion in comparison to control and no morphological change are observed. The effects of metformin on neutrophils adhesion in the presence of glucose concentration were minor, did not show any significant effect.

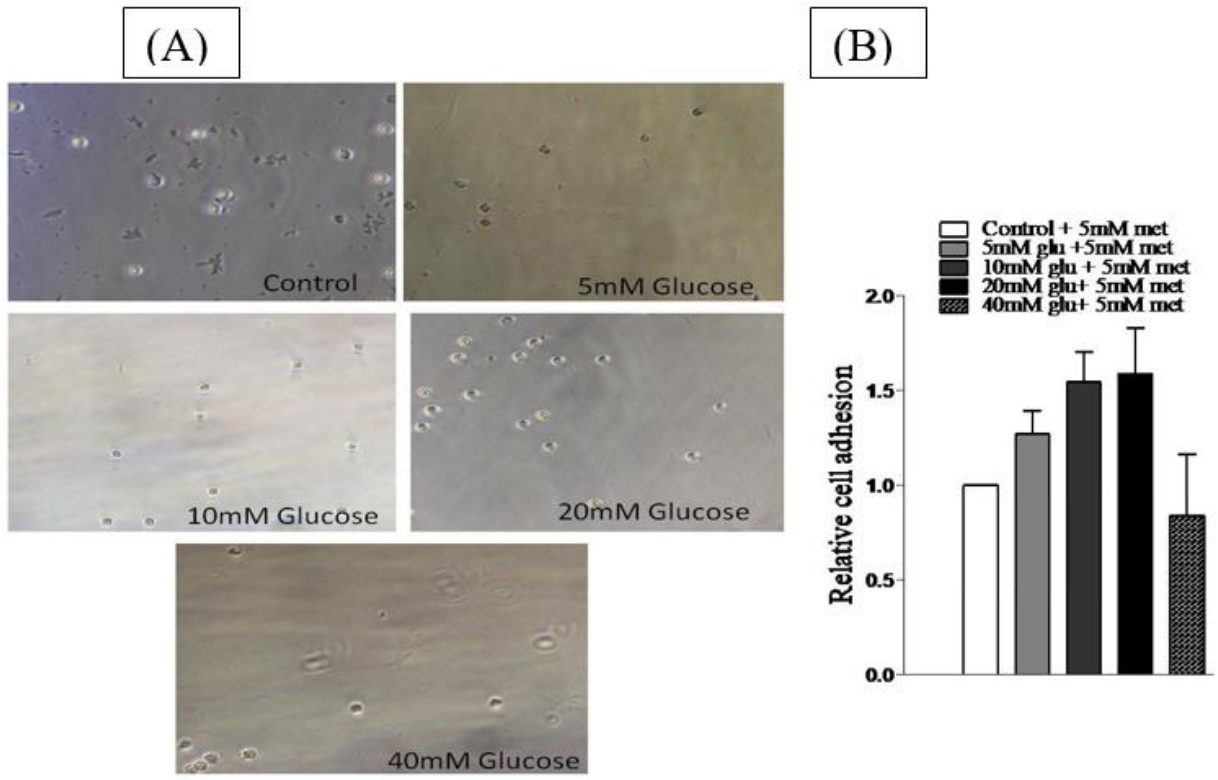

Figure 5-Effect of Metformin (5mM) on neutrophil adhesion.

(A) Representative image of neutrophil treated different glucose concentration in the presence of metformin (5mM) **(B)** Bar graph data is represented as the mean ± SEM.

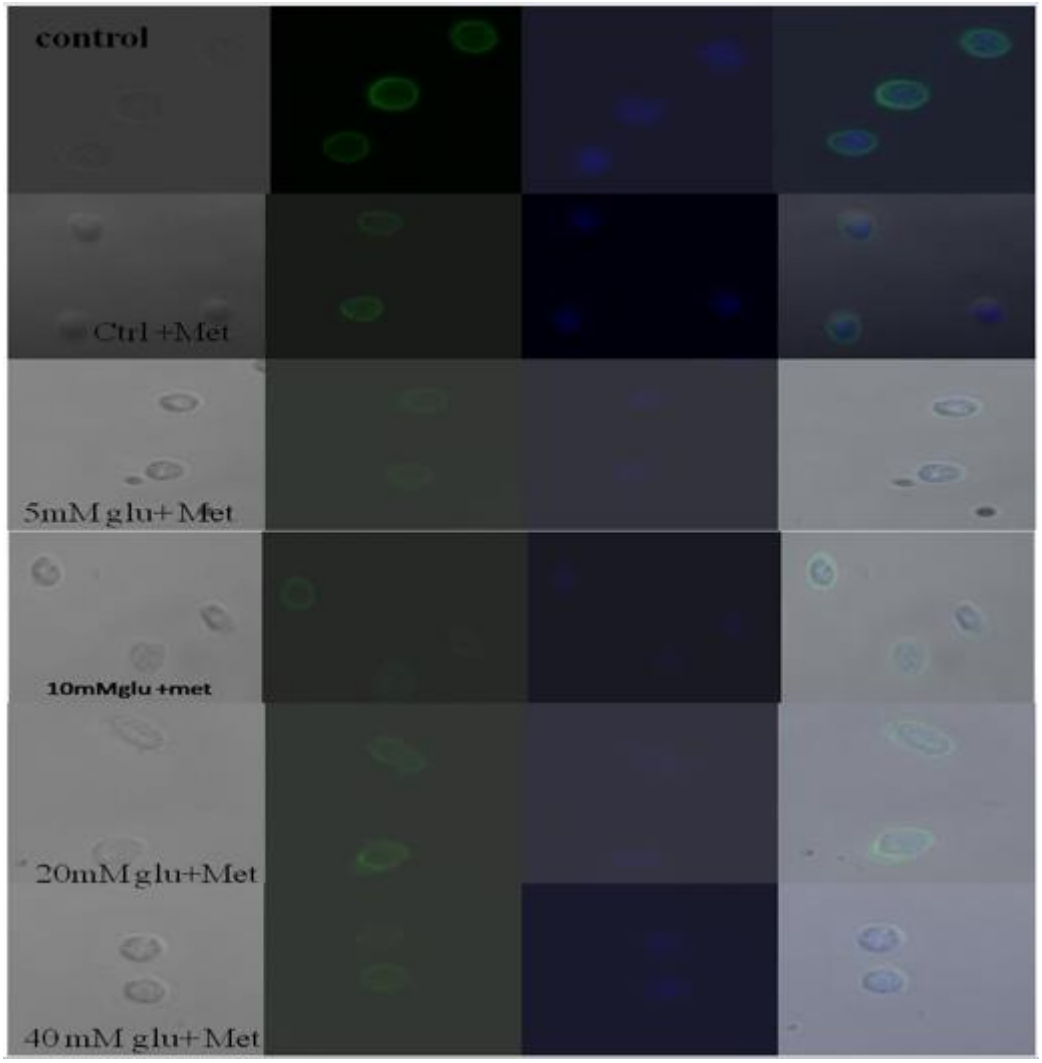

Figure 6- Effect of metformin on glucose induced CD11b expression on neutrophils. (A) Neutrophils is treated with different glucose concentration (5mM, 10mM, 20mM and 40mM) in the presence of metformin (5mM). Images were captured using Leica microscope. CD11b staining is in green channel, blue colour is demonstrating nucleus.

Effects of Pioglitazone on Neutrophil Adhesion -

I further found effect of other drug, pioglitazone on neutrophils. PMNs were incubated with pioglitazone (10μM) for 30 minutes in the presence or absence of fMLP (10μM). Light microscopy showed that pioglitazone treated cells do not show any morphological alteration. In the presence of fMLP, shows morphological changes as compared to control. Pioglitazone exhibited a tendency to suppress the fMLP-induced increase the adherence but it do not show any significant effect.

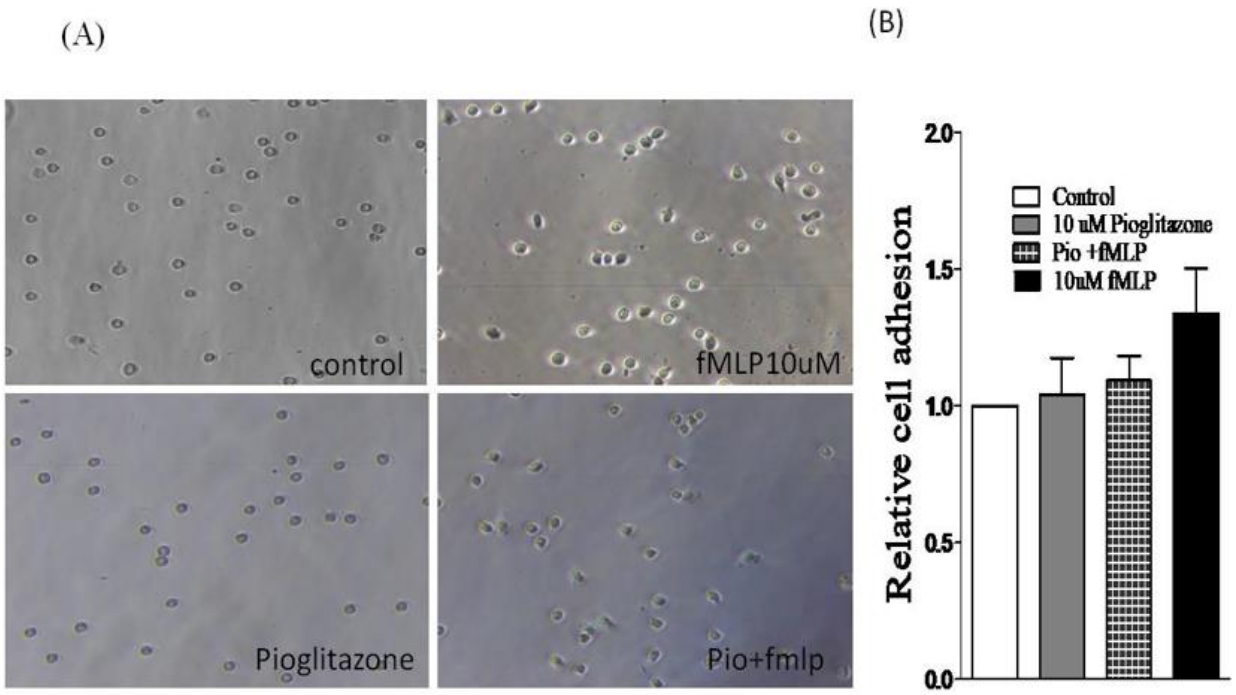

Figure 7-Effect of Pioglitazone on neutrophil adhesion.

(A) Representative image of neutrophil treated with piogiltazone**(B)** PMNs were incubated with pioglitazone (10µM) for 30 minutes in the presence or absence of fMLP. Data is represented as the mean ± SEM.

Effect of anti-diabetic drugs on neutrophil apoptosis:-

To identified the effect of metformin on neutrophils apoptosis. PMNs were incubated with Metformin (5mM) and fMLP (10µM) for 18 hours at 5% CO_2 incubator. The neutrophils apoptosis has analysed using Propidium iodide (1µg/ml) and Annexin V FITC staining. By Using this method, I analyzed the earlier phase and later phase of apoptosis. then incubate the neutrophil with fMLP (10µM) for 18 hours shows increased apoptosis as compared to control and incubate with metformin (5mM) for 18 hours shows increased survival rate and decrease neutrophil apoptosis.

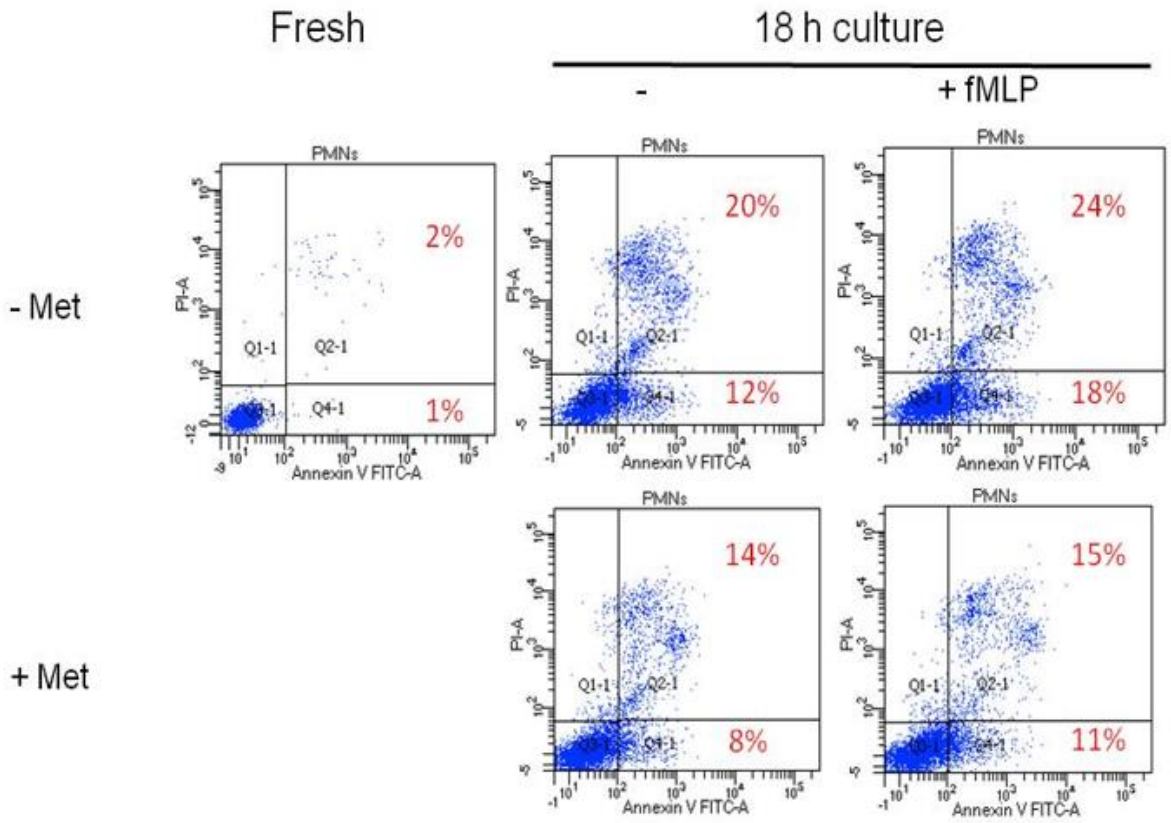

Figure 8:-Apoptosis of neutrophils after exposure of metrormin (5mM) and fMLP(10µM).

Neutrophils has incubated for 18 hours at 5% CO_2 incubator. The percentage of Annexin V or propidium iodide positive cell was determined by flow cytometry. Representative dots plot for phosphatidylserine externalization by apoptotic cell as well as viable cell (region Q3), early apoptotic (region Q4), late apoptotic (region Q2) and necrotic cells (region Q1).

DISCUSSION

Diabetes mellitus is a metabolic disorder characterized by the increased glucose levels in the body. Hyperglycemia or increased glucose concentration in blood inhibits the neutrophil migration, phagocytosis and microbial killing and apoptosis. Extracellular stimuli such as bacterial derived fMLP shown to induce neutrophil adhesion[20]. Apoptosis (program cell death) is a mechanism for neutrophil clearing during and post inflammation. Apoptotic signaling in neutrophils is also related to the dysregulation of no. of neutrophil functions including phagocytosis and chemotaxis. Apoptotic neutrophils are identified and phagocytosed by the macrophages which do not shows to decrease proinflammatory mediators. Apoptotic neutrophils show downregulation of expression of selectin and selectin

ligands while expression of CD11b/CD18 was maintained. The other aim of this study was to evaluate the effects of anti-diabetic drug on neutrophil apoptosis. The earlier stage of apoptosis is identified by the morphologically and later phage of apoptosis is determined by the DNA binding dye propidium iodide after the treatment with LPS, fMLP and antidiabetic drugs. Our study demonstrates that fMLP increased the neutrophil apoptosis and thus decreased the neutrophils survival. While metformin treatment reduced the neutrophils apoptosis after 18 hours treatment [21]. We have confirmed that increased neutrophils apoptosis after fMLP stimulation and decrease the neutrophils apoptosis after treatment with metformin in all the examined group.

CONCLUSION

In conclusion, we demonstrate that fMLP treated cell shows increased cell adhesion and metformin treated cell shows no effect on cell adhesion after 30 min incubation. In our study, we have found that induction of high glucose (5mM, 10mM and 20mM) result in increased the cell adhesion and further increased concentration of glucose (40mM) led to inhibits the cell adhesion. It has been identified that high glucose concentration can increase the various physiological process in many cell types. Some studies shows that high glucose concentration can stimulate a signaling pathway which is conducted by protein kinase C. Most of the studies have shown that metformin decreased the cardio vascular disease in type 2 diabetes mellitus but mechanism is remains unknown for this. In the next study, LPS and fMLP treated cell show increased neutrophil apoptosis and decrease the cell survival after 18 hrs treatment. Metformin treated cell indicate increased cell survival rate and decrease the neutrophil apoptosis after 18 hrs treatment. We found that increased cell apoptosis and decrease cell survival after 18 hrs fMLP treatment. Metformin treated cell shows protective effect and increased survival rate and decrease the cell apoptosis. Overall, metformin seems to protect neutrophil from apoptosis, while no effect is observed on adhesion.

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