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Effect of Diclofenac on Plasma Glucose level, Insulin Resistance, Inflammatory Markers and Hepatocytes in Diabetic Albino Rats

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Abstract

Background and aim of the study: diabetes was proposed to be an inflammatory disease. Growing evidence has pointed to a correlation between various proinflammatory cytokines, insulin resistance (IR) and type 2 diabetes (T2DM).

Materials and Methods: This study was carried out on one hundred Albino rats, distributed into four groups. Group I: control group, Group II: diabetic rats with no treatment, Group III: diabetic rats treated with Glipenclamid and Group IV: diabetic rats treated with Diclofenac sodium. Blood samples were taken and the following biochemical parameters were done: fasting blood glucose (FBG), serum insulin, aspartate transaminase (AST), Alanine transaminase (ALT), serum Alkaline Phosphatase (ALP), serum protein, serum albumin, serum triglyceride (TGs), serum cholesterol level (TC), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Tumor Necrosis Factor (TNF-α) and C-Reactive Protein (CRP). HOMA IR and HOMA B were calculated. Liver samples from all rats were obtained and stained with Hematoxylin and Eosin, Masson's trichrome and Periodic acid–Schiff (PAS) for histological examination.

Results: Declofenac caused significant lowering in FBG, lipid profile, TNF-α level, CRP, increased insulin secretion with improved IR and beta cell function compared to the diabetic group. There was a positive correlation between HOMA-IR and CRP; HOMA-IR and serum TNF-α. Liver of diabetic rats showed periportal fibrosis, vacuolated cytoplasm and nuclei and glycogen deposition. These changes improved markedly in Glibenclamide treated groups while liver of Declofenac treated group revealed parenchymal cell necrosis, sinusoidal dilatation with some pyknotic nuclei and marked glycogen deposition.

Conclusions: inflammatory pathways may play an important part in IR of T2DM. Therefore, antinflammatory drugs may have a role in diabetes therapy through improving IR because of its insulin-sensitizing and anti-inflammatory properties.

Key Words: Diabetes Mellitus, Inflammation, Insulin Resistance; Diclofenac Sodium, Glibenclamide Histopathological Liver Changes.

Introduction

Diabetes mellitus (DM) is possibly the world's fastest growing metabolic disease, so there is a great need for more appropriate therapies. (1) A lot of evidence suggests that insulin resistance (IR), defined as a state of reduced insulin action in peripheral tissues, such as the skeletal muscle, adipose tissue, and liver plays a critical role in the development and onset of T2DM. (1, 2) A number of epidemiological studies have demonstrated that IR exists in the prediabetic state, often predating the onset of DM by many years. In the presence of IR, a normal beta cell will increase production of insulin, and as long as the compensatory hyperinsulinemia is adequate to overcome the IR, glucose tolerance remains relatively normal. In patients destined to develop T2DM, the beta cell compensatory response fails, and relative insulin insufficiency develops, leading to impaired glucose tolerance and eventually frank T2DM.(3)

T2DM and the IR syndrome have recently been hypothesized to represent an acute phase response.(4) This is in part due to the strong correlation between elevations in the local and circulating proinflammatory cytokines, TNF, interleukin-1 (IL-1), interferon, and IL-6 and IR.

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[5] It is well known that these cytokines are elevated in cases of cancer, cachexia and severe infection where IR frequently accompanies these clinical conditions. Importantly, the proinflammatory cytokines have also been shown to be elevated in T2DM.[6] In recent years, more evidence has emerged that proinflammatory cytokines can cause sustained development of IR and anti-inflammatory medications may reverse the process of it. [7] A number of epidemiological studies and animal research have shown a correlation between IR and inflammatory markers such as TNF-α, CRP and IL-6, suggesting that inflammation may be directly involved in the pathogenesis of IR.[8]

TNF-α, produced by adipocytes and macrophages, has been implicated to play an important role in the cascade of inflammation, systemic IR, reduced β-cell secretion of insulin, and thus T2DM. [9] There is evidence indicating that TNF-α induces the overproduction of very low density lipoprotein (VLDL) particles, which might explain its direct relationship with plasma TGs. [10] CRP is a key inflammatory factor produced by the liver in response to an acute infection or inflammation and it is regulated by IL-6, IL-1, and TNF-α. [11]

Recent study indicated that aspirin might be an insulin-sensitizing agent and it may be used to reverse hyperglycemia and hyperinsulinemia by improving IR.[12] However, the cellular and molecular mechanisms of aspirin in improving insulin resistance in T2DM have not been well elucidated.

In the present study, we examined the effect of Declofenac on inflammatory markers, IR, and hepatic cellularity in comparison to strong antidiabetic drug Glibenclamide in rat model of T2DM induced by Alloxan.

Material and Methods

Animal Study

One hundred adult male albino rats 10-12 weeks of age with body weight ranging between 180-200 gm were used in the current work, 25 of them were used as healthy control group. Animals were obtained from the Laboratory Animal Unit, King Fahd Medical Research Centre, King Abdulaziz University, Jeddah. All experiments were taken place at the research laboratories, College of Medicine, Taif University. Animals were housed individually in clean rodent cages, a room at relative humidity not less than 30% and not exceeding 70%, at room temperature 22 °C - 30 °C, with artificial lighting in a sequence 12 hours light and 12 hours dark. Animals were fed on conventional laboratory animal diet for rats with an unlimited supply of drinking water. Animals were randomly selected, marked to permit group identification. The experimental and feeding protocols of the animals used in this study was approved and performed according to the guidelines of Animal House and Ethical Standards of College of Medicine, Taif University, KSA. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health as well as the guidelines of the Animal Welfare Act.

Induction of DM was done on 75 rats by giving subcutaneous injection freshly prepared Alloxan solution 120 mg/kg (powder from BDH chemical LTD, England), dissolved in acetate buffer (pH 5.5) prepared immediately before use. After an overnight fasting then 48 hours later, blood glucose level was determined by Glucometer for all animals. Rats with blood glucose level ranging from 180 to 250 were considered diabetic.

The rats were distributed randomly into four groups (each group containing 25 rats) as follows:

Group I: Healthy control group.

Group II: diabetic rats with no treatment as a diabetic control.

Group III: diabetic rats treated with Glipenclamide

Group IV: diabetic rats treated with Diclofenac sodium.

Drug Administration

- Diclofenac sodium given orally to rats
  - Dose: (150 mg/day).
- Glibenclamide.
  - Dose: 5mg/kg

Follow-up

Animals follow-up included changes in clinical and biochemical parameters for 4 weeks.

Biochemical assays

Blood samples were obtained, and then centrifuged at 4000 (rpm) for 10 min at 4°C and supernatant kept at -70°C for further biochemical measurements. Blood samples were taken and biochemical studies were done to assess the following biochemical parameters: blood glucose level (BG), serum insulin level, AST, ALT, ALP, serum albumin concentration, serum protein, serum globulin, serum TGs, serum TC, HDL, LDL, TNF-α was measured by radioimmunoassay with an automatic biochemical analytic instrument using TNF-α ELISA Kits, Thermo Scientific and CRP (The Invitrogen CRP ELISA Kit, Camarillo, CA).

Homeostasis Model Assessment: HOMA is an arithmetic way of deriving indices of pancreatic endocrine function HOMA- β and HOMA-IR from fasting plasma samples. [13] This model assumes that plasma glucose and insulin in the fasting state is controlled by a feedback loop between the pancreas, liver, and insulin-sensitive and insulin-insensitive peripheral tissues. HOMA correlates well with and is validated against the gold standard methods of assessment of these functions, such as the euglycemic hyperinsulinemic clamp. [14]

HOMA-IR and HOMA- β are derived using the formulae:

HOMA-IR = [glucose (mg/dL) × insulin (mU/l)] / 405

HOMA-β = [360 × insulin (mU/l)] / [glucose (mg/dL) – 63)]

IR is insulin resistance and % β is the β-cell function. Glucose and insulin are both during fasting. In an "ideal" reference population of young, healthy subjects HOMA- β and HOMA-IR are 100% and 1 (arbitrary units), respectively.

Histological studies

After 4 weeks, all animals were fasted overnight, and then weighted. The animals were killed by decapitation. An equal liver sample from each rat were obtained and fixed in 10% neutral buffered formal saline, dehydrated in ascending grades of alcohol and cleared in Benzol. Samples from each group were embedded in paraffin with a melting point between 55 °C and 56 °C for 4 hours and then paraffin blocks were prepared. Paraffin sections were made at 5 µm and stained with:

1- ***Hematoxylin and Eosin.*** for normal histology and histopathology.
2- ***Masson's trichrome*** for the detection of collagen fibers in tissues. Collagen fibers were stained blue and the nuclei were stained black and the background was stained red.
3- ***Periodic acid–Schiff (PAS)*** to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. The Schiff reagent give a purple-magenta color, followed by light microscopy for demonstrating any histological changes. [15]

Statistical analysis

Data were analyzed using SPSS version 20. The data were expressed in means ± SDs. The comparison of quantitative data was performed by independent t-test or Mann Whitney test according to normality of distribution for independent variables consisting of two groups and by ANOVA and Tukey-Kramer multiple comparisons test according to normality of distribution for independent variables consisting of more than two groups. A p-value of <0.05 was considered as statistically significant. [16]

Results

Tables (1) revealed comparison between different studied groups as regard liver function tests after induction of DM by Alloxan, there was a significant increase in AST, ALT and ALP with marked increase in Declofenac group with no significant difference between DM control and Glibenclamide groups. There was insignificant decrease in serum protein and serum albumin in diabetic groups. Serum globulin insignificantly increased in diabetic groups.

Table (2) revealed comparison between different studied groups as regard insulin and FBG level. Serum insulin level significantly decreased in diabetic control group compared to healthy control group with significant increase in insulin level in Glibenclamide and Declofenac groups. FBG level significantly increased in diabetic control group compared to healthy control group and significantly decreased in Glibenclamide and Declofenac groups with no significant difference between them. HOMA IR

significantly increased in diabetic control group compared to healthy control group and significantly decreased in Glibenclamide and Declofenac groups with no significant difference between them, while HOMA B significantly decreased in diabetic control
group compared to healthy control group and significantly increased in Glibenclamide and Declofenac groups compared to diabetic group with no significant difference between them.

Table (3) revealed comparison between different studied groups as regard lipid profile, there is a significantly increased levels of TC, TGs and LDL with significant decrease in HDL level in all diabetic groups compared to healthy control group. Serum TC, TGs and LDL significantly decreased in Glibenclamide and Declofenac groups but their levels were still higher compared to healthy control group, while HDL significantly increased in Glibenclamide and Declofenac groups with insignificant difference between treated groups.

Table (4) showed comparison between different studied groups as regard inflammatory markers, there is a significant increase in CRP and TNF in diabetic groups compared to healthy control group. CRP significantly decreased in Glibenclamide and Declofenac groups with marked decrease in Declofenac group. TNF significantly decreased in Glibenclamide and Declofenac groups with marked decrease in Declofenac group with no significant difference between healthy control, Glibenclamide or Declofenac groups.

Table (5), Figure 4 and 5, revealed significant positive correlation between IR and inflammatory markers (CRP and TNF) in different studied groups.

Figure (1), Hematoxylin and Eosin of liver rats revealed (A) normal histological architecture of liver, normal lobular structure with central vein and hepatocytes (B) Liver of diabetic control rats showing periportal fibrosis, vacuolated cytoplasm and cellular infiltration. (C) Liver of diabetic rats treated with Glibenclamide showing somewhat normal liver cells and nuclei (D) Liver of diabetic rats treated with Declofenac showing parenchymal cell necrosis, sinousoidal dilatation, some pyknotic nuclei and cytoplasmic infiltration.

Figure (2): Trichrome stain of liver rats (A) normal histological architecture of liver with normal lobular structure is seen (B) Liver of diabetic rats shows periportal fibrosis and cellular infiltration (C) Liver of diabetic rats treated with Glibenclamids showing all hepatic sinusoids becomes slightly thinner and with almost a normal liver architecture (D) Liver of diabetic rats treated with Declofenac showing periportal fibrosis and vacuolated cytoplasm.

Figure (3), PAS stain of liver normal glycogen distribution in the liver cell (B) Liver of diabetic rats showed glycogen deposition with vacuolated cytoplasm. (C) Liver of diabetic rats treated with Glibenclamide showing all hepatic cells are large and red stained with almost a normal liver architecture (D) Liver of diabetic rats treated with Declofenac showing changes in all hepatocytes. These cells became large with red stained granules. The cytoplasm showed glycogen deposition with vacuolated cytoplasm. compare group treated by Glibenclamide and Declofenac revealed that Glibenclamide is more effective than Declofenac in improving liver abnormalities occurred in diabetic liver rats.

Discussion:

Severe hyperglycemia in diabetic rats recorded in the present work can be considered as a direct reflex to the marked hypoinsulinemia caused by the selective destructive cytotoxic effect of Alloxan on the β-cells of the pancreas which has a direct effect on their membrane permeability by causing failure of ionic pumps and increased cells size. [(17)] Our results revealed FBG lowering effect in the treated groups after the 4 weeks with no significant difference between Glibenclamide and Declofenac treatment. Also, insulin secretion significantly decreased in diabetic control group and significantly increased in Glibenclamide and Declofenac treated groups compared with the non-treated group ($p= 0.0001$).

HOMA IR significantly increased in diabetic control group and significantly decreased in Glibenclamide and Declofenac treated groups as compared with the non-treated group, with marked decreased in Declofenac group ($p= 0.001$) while HOMA B significantly decreased in diabetic non treated groups and significantly

increased in Glibenclamide and Declofenac treated groups as compared with the non-treated group with marked increased in Glibenclamide group (p= 0.001). IR is not only an initiating pathogenic marker but also a major factor that promotes the development of T2DM. Overt diabetes is thought to be preceded by a long period of IR, during which sufficient insulin is produced to maintain normal or near-normal glucose tolerance. However, this chronic compensatory insulin hypersecretion to overcome tissue insensitivity can itself finally lead to pancreatic beta cell failure and overt hyperglycemia. [18] In the present study, diabetic control rats showed significant increase in FBG, and HOMA-IR values with decreased insulin secrtion compared to the normal control group, which indicated development of IR. In the present study, the diabetic control group showed significant increase in TC, TG, LDL and decrease HDL compared to healthy control group which significantly improved in Glibenclamide and Declofenac treated groups compared to non-treated group and to (p= 0.0001) with no significant difference between the two groups. This comes in agreement with that of other authors. This may be attributed to stimulation of these drugs to the most aspects of carbohydrate metabolism, including rapid uptake of glucose by the cells, enhanced gluconeogenesis, increased rate of absorption from the gastrointestinal tract and even increased insulin secretion with its result of secondary effects on carbohydrate metabolism. [19]

Our results revealed a significant increase in AST, ALT, ALP and globulin with significant decrease in serum protein and albumin in the diabetic group. Also, diabetic liver showed periportal fibrosis, vacuolated cytoplasm and cellular infiltration. There are no significant changes in serum protein or serum between healthy control, diabetic control and Glibenclamid groups. A non significant decrease in ALT, AST and ALP values were found in Glibenclamide treated group compared to diabetic non treated group, while it persistently increased in Declofenac treated group (p= 0.001). This may be attributed to the dose of Diclofenac. Zeynab and Ibrahim [20] found that administration of Diclofenac sodium at high dose induced some adverse effects on hematological, biochemical, oxidative parameters as well as histology of liver and kidney. This could be attributed to oxidative stress induced by the drug. However, these effects were reversible. They also found a significant decrease in serum total protein and albumin levels and significant increase in aminotranseferases, ALP, urea and creatinine levels.

In addition there were a significant reduction in reduced glutathione (GSH) levels and significant increase in malondialdehyde (MDA) content in liver and kidney homogenates. Histopathological alterations were found in livers and kidneys. However, Diclofenac sodium at dose of 6.75 mg / kg body weight induced non significant changes in the previous parameters. Liver of diabetic rate shows periportal fibrosis, vacuolated cytoplasm and nuclei and glycogen deposition. These changes improved markedly in Glibenclamide treated groups while liver of Declofenac treated group revealed parenchymal cell necrosis, sinusoidal dilatation with some pyknotic nuclei and cytoplasmic infiltration and marked glycogen deposition. Histological study of the liver of the diabetic rats (30 day treatment) showed histological improvement of liver texture especially in the diabetic group. Our study revealed increased CRP and TNF in diabetic group which significantly decreased in Glibenclamide and Declofenac treated groups compared to non-treated group (p= 0.0001) with insignificant difference between treated and healthy control groups. The decrease was more marked in Declofenac group with a positive correlation between IR and CRP and TNF. Pickup et al. [21] first proposed that diabetes is an inflammatory disease state. Growing evidence has pointed to a correlation between various proinflammatory cytokines, IR and T2DM.[22] Recent data have revealed that adipocyte-derived TNF-α, an inflammatory cytokine produced mainly by monocytes and macrophages was increased in the IR states of obesity and T2DM. [23] Thus, TNF-α has been recognized as an important mediator for IR by impairing insulin signaling. TNF-α can decrease glucose uptake and utilization of peripheral

tissues by targeting insulin signaling pathways and glucose transporter 4 (GLUT4).

The mechanism of the decrease is due to stimulation of serine phosphorylation of IRS1 and IRS2, inhibiting tyrosine kinase activity of the insulin receptor and phosphatidylinositol-3-kinase (PI3K) signaling pathways.[24] NF-κB is a proinflammatory master switch that controls the production of a host of inflammatory markers and mediators, including TNF-α, IL-6, CRP, and PAI-1. [25] It was found that NF-κB can be activated by sustained high blood glucose. [26] Finally, TNF-α is not only induced by NF-κB, but also is a strong activator of NF-κB. [27] The liver is the key organ of IR in T2DM. Decreased hepatic insulin sensitivity may lead to increased hepatic gluconeogenesis, postprandial hyperinsulinemia and increased formation of TGs in the liver cells. In our study, we found that hepatocytes became larger and showed glycogen deposition in the cytoplasm. [28]

Sun et al. [23] stated that lipid accumulation in the liver leads to hepatic 'inflammation' through NF-κB activation and downstream cytokine production, upregulates TNF-α production and secretion. Given that the link between IR and inflammation, limiting inflammation and reducing levels of inflammatory markers may be a promising therapeutic strategy. In our study, we found that the Declofenac-treated group showed significant increased of serum insulin and reduction HOMA-IR, indicating that it can improve IR. Also, we found that Declofenac had significant therapeutic effect on improving dyslipidemia and hyperglycemia associated DM. **Sun et al.** [23] found the same results with Asprin treated rats with no effects on associated hyperglycemia and dyslipidemia. In their study, TNF-α levels were also decreased compared to diabetic groups. They suggest that aspirin improves IR by inhibiting hepatic NF-κB activation and TNF-α level, so NF-κB cannot control the downstream expression of inflammatory cytokines, which reduce IR. [29]

Conclusion:

The inflammatory pathways may play an important part in IR of T2DM. Therefore, antinflammatory drugs may have a role in diabetes therapy through improving IR because of its insulin-sensitizing and anti-inflammatory properties. Declofenac used in this study have harmful effect on the liver in the form of parenchymal cell necrosis, sinousoidal dilatation, cytoplasmic infiltration, periportal fibrosis and vacuolated cytoplasm.

Recommendation:

Wide scale studies with different anti-inflammatory drugs at different doses for long term duration are recommended to be done on newly discovered diabetic patients to clarify their roles and determine save and effective dose.

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Table (1): Comparison between different studied groups as regard liver function tests.

	Healthy control		DM control		Glibenclamide		Declofenac		P
	M	SD	M	SD	M	SD	M	SD	
ALT u/l	33.9	8.7	44.1	1.3	41.9	0.96	73.9	1.8	0.0001
	NS between DM control and Glibenclamide groups								
AST u/l	45.6	1.1	65.1	2.6	64.4	1.7	80.3	0.73	0.0001
	NS between DM control and Glibenclamide groups								
ALP IU/l	47.7	3.3	81.4	3.31	79.52	1.52	90.3	7.72	0.0001
	NS between DM control and Glibenclamide groups								
Total protein gm/dl	6.59	0.38	6.57	0.39	6.61	0.35	5.66	0.36	0.4216
Albumin gm/dl	4.22	0.19	3.94	0.18	3.92	0.16	3.24	0.45	0.4036
Glublin gm/dl	3.08	0.41	3.19	0.06	3.48	0.48	4.13	1.78	0.7913

Table (2): Comparison between different studied groups as regard insulin and blood sugar level.

	Healthy control		DM control		Glibenclamide		Declofenac		P
	M	SD	M	SD	M	SD	M	SD	
Insulin mU/l	42.06	1.9	23.55	3.07	32.35	3.03	28.11	2.95	0.0001
FBG mg/dl	115.4	8.9	332.5	12.03	130.2	7.19	138.9	2.47	0.0001
	NS between Glibenclamide and Declofenac groups								
HOMA IR	5.44	0.2	6.44	0.81	4.25	0.32	3.86	0.4	0.0001
	NS between Glibenclamide and Declofenac groups								
HOMA B %	195.56	31.57	23.77	2.35	126.98	15.61	107.33	9.32	0.0001
	NS between Glibenclamide and Declofenac groups								

Table (3): Comparison between different studied groups as regard lipid profile.

	Healthy Control		DM control		Glibenclamide		Declofenac		P
	M	SD	M	SD	M	SD	M	SD	
TC mg/dl	90.89	4.76	121.4	7.4	103.3	2.2	103	1.1	0.0001
TG mg/dl	118.1	5.04	152.6	13.56	131.2	7.99	132.5	7.66	0.0001
HDL mg/dl	47.09	0.61	31.78	1.64	42.89	1.36	40.11	1.9	0.0001
	NS between Glibenclamide and Declofenac groups								
LDL mg/dl	43.34	3.76	65	7.41	46.31	3.63	46.11	3.79	0.0001

Table (4): Comparison between different studied groups as regard Inflammatory Markers.

	Healthy control		DM control		Glibenclamide		Declofenac		P
	M	SD	M	SD	M	SD	M	SD	
CRP mg/L	0.88	0.12	5.22	0.37	3.2	0.3	1.9	0.3	0.0001
TNF pg/mL	7.38	1.19	19.84	0.73	8.12	2.33	6.12	0.89	0.0001
	NS between healthy control, Glibenclamide or Declofenac groups								

Table (5): Correlation between insulin resistance and inflammatory markers in different studied groups.

	IR	P
CRP	**r =** 0.4578	0.0030
TNF	**r** = 0.7407	0.0001

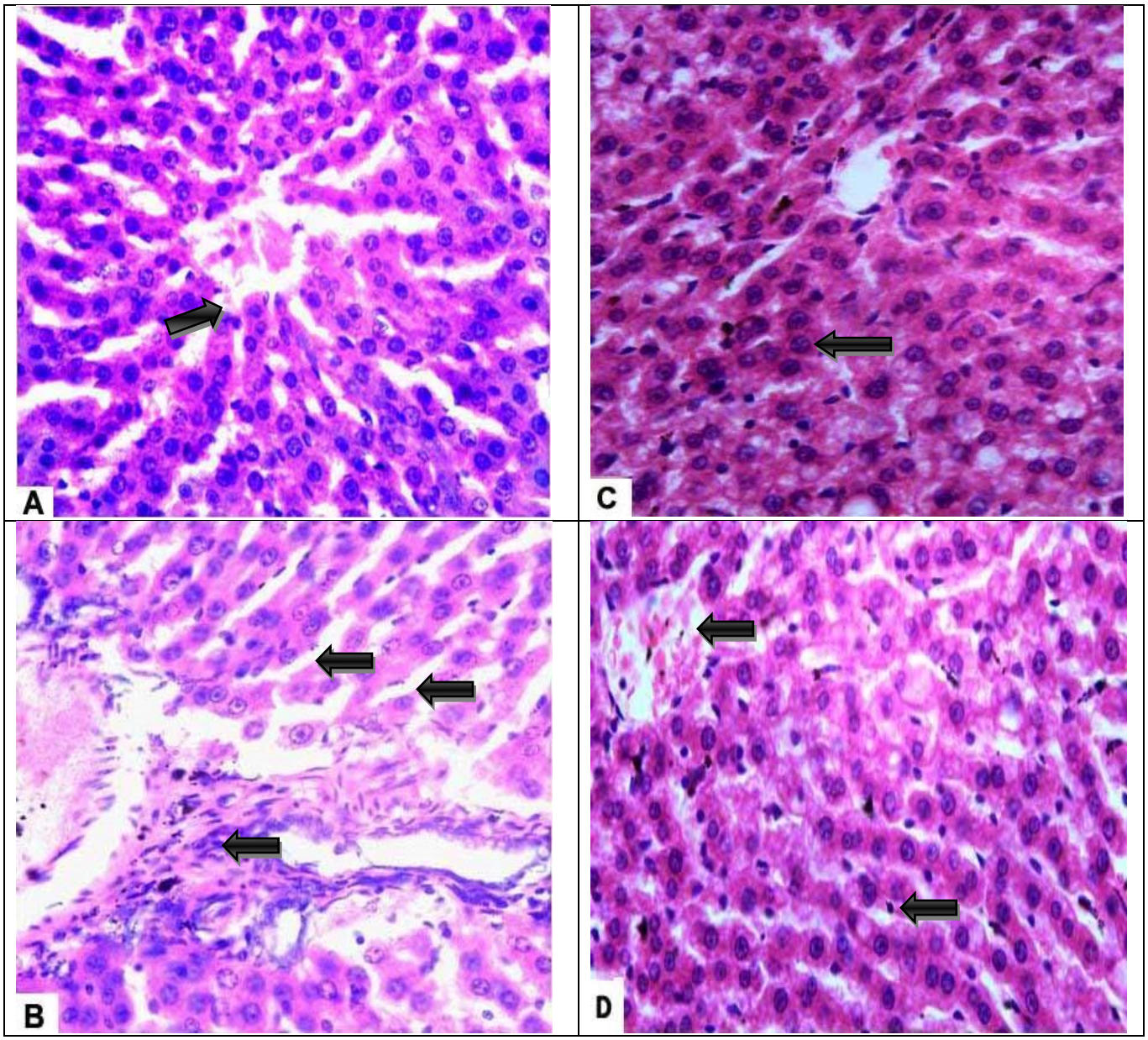

Fig (1): Photomicrograph of liver rats (A) Normal histological architecture of liver, normal lobular structure with central vein on hepatocytes (B) Liver of diabetic rat showing periportal fibrosis, vacuolated cytoplasm and cellular infiltration. (C) Liver of diabetic rat treated with Glibenclamid showing somewhat normal liver cells and nuclei (D) Liver of diabetic rat treated with Declofenac showing parenchymal cell necrosis, sinousoidal dilatation, some pyknotic nuclei and cytoplasmic infiltration. (Hx& E X400).

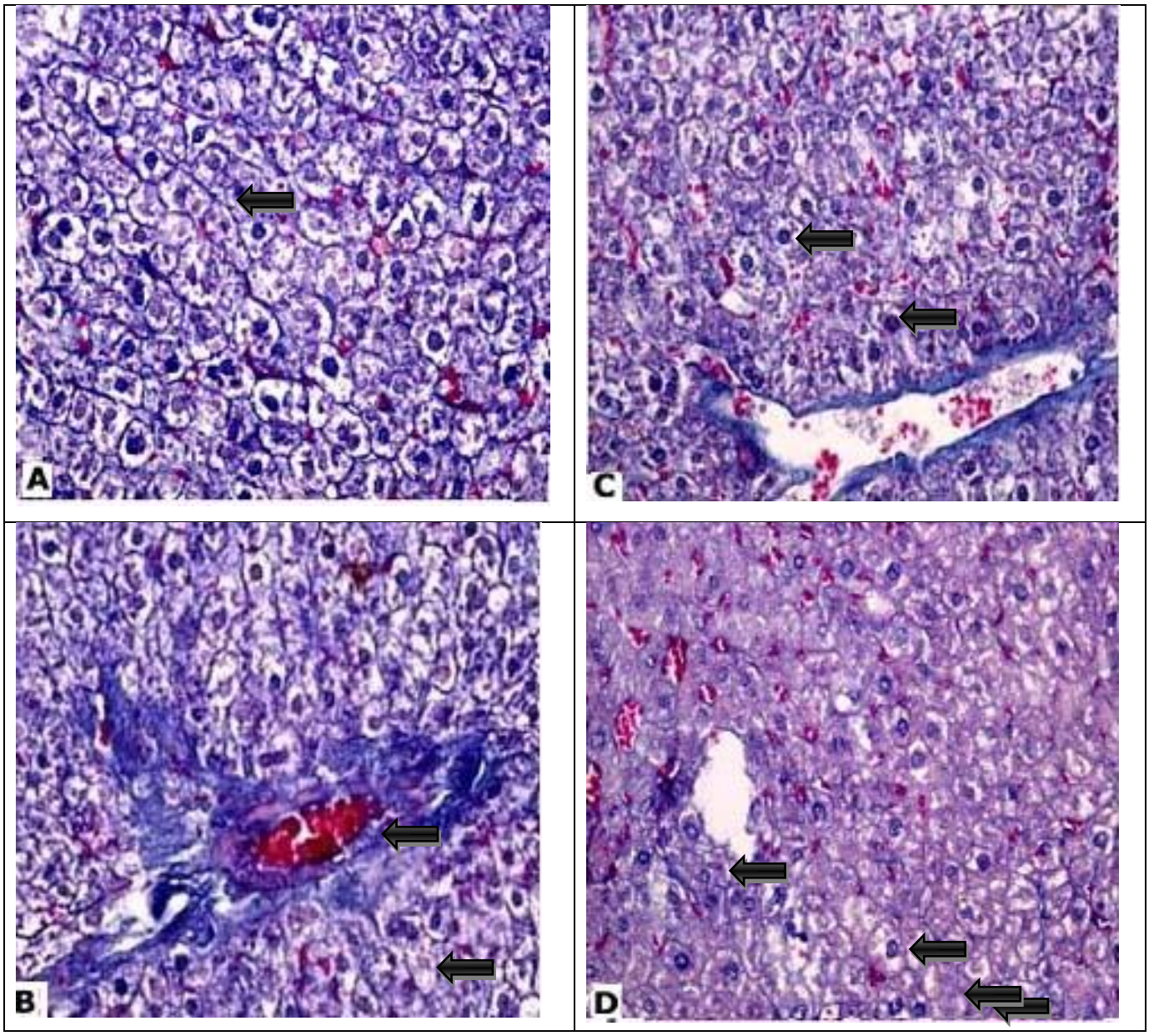

Fig (2): Photomicrograph of liver rats (A) normal histological architecture of liver with normal The normal collagen distribution (B) Diabetic liver shows periportal fibrosis and cellular infiltration (C) Liver of diabetic rat treated with Glibenclamide showing all hepatic sinusoids becomes slightly thinner and with almost a normal liver architecture (D) Liver of diabetic rat treated with Declofenac showing periportal fibrosis and vacuolated cytoplasm. (Trichrome stain X4001).

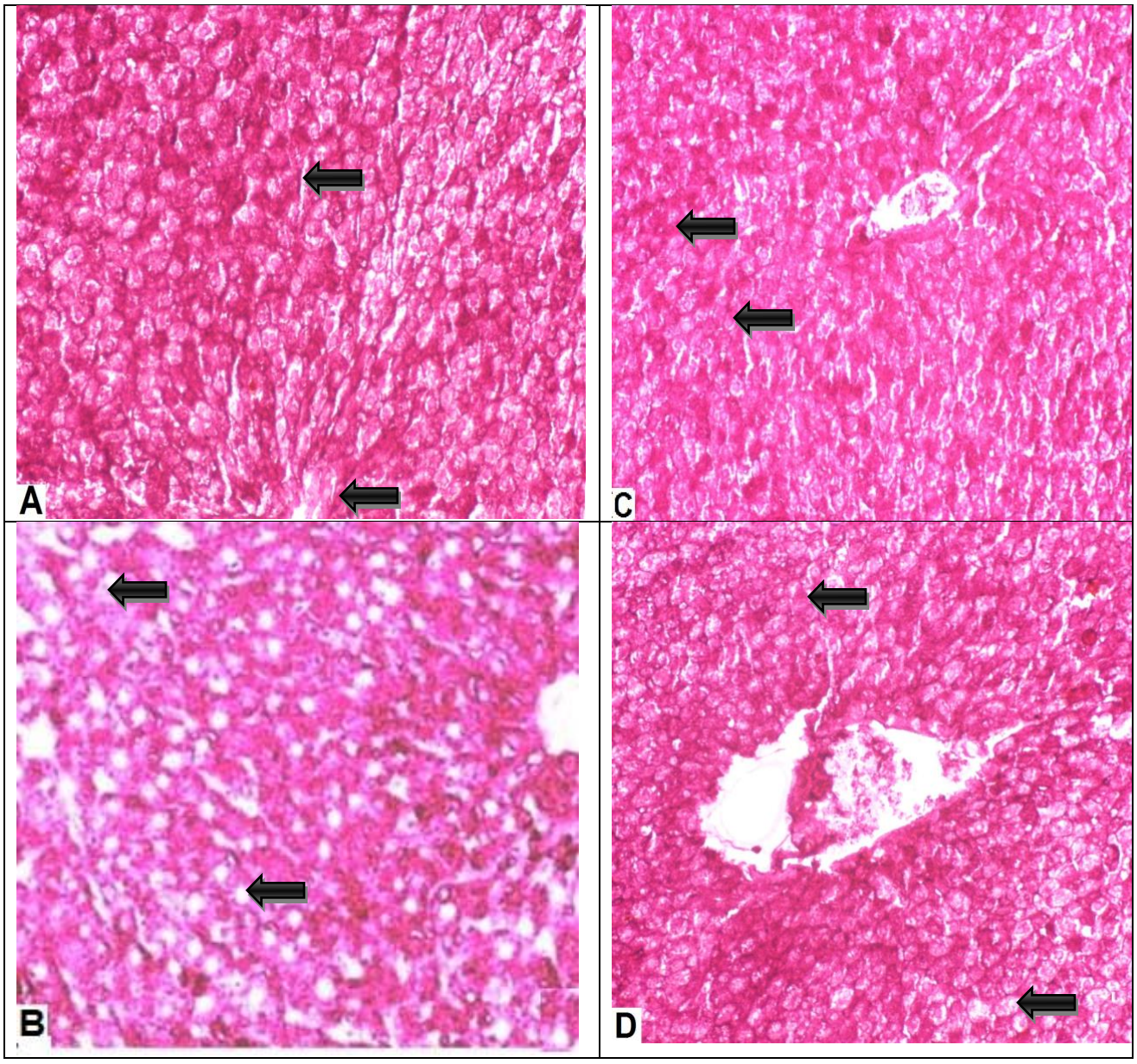

Fig (3): Photomicrograph of liver rats (A) Normal histological architecture of liver and normal glycogen distribution in the liver cell (B) Diabetic liver showed glycogen deposition with vacuolated cytoplasm. (C) Liver of diabetic rats treated with Glibenclamide showing all hepatic cells red stained and with almost a normal liver architecture (D) Liver of diabetic rats treated with Declofenac showing all hepatocytes become red stained granules cytoplasm showed glycogen deposition with vacuolated cytoplasm. (PAS stain X400)

Figure (4): Correlation between Insulin Resistance (IR) and Tumor Necrosis Factor (TNF).

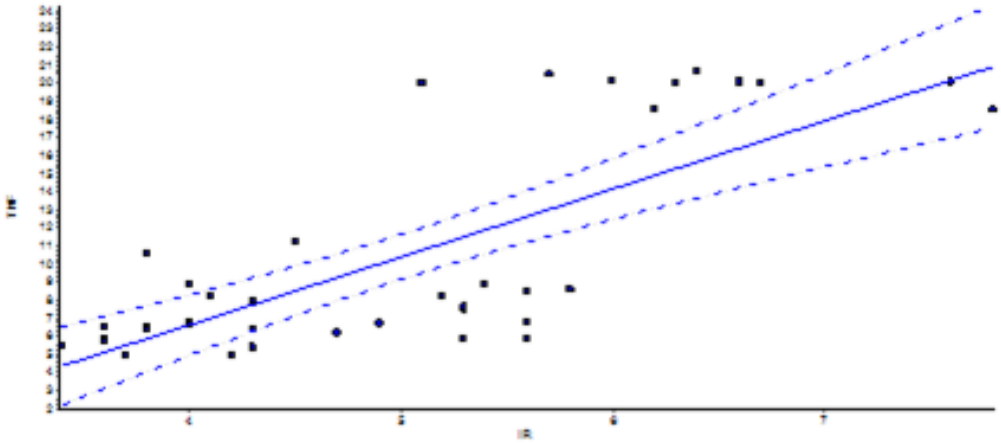

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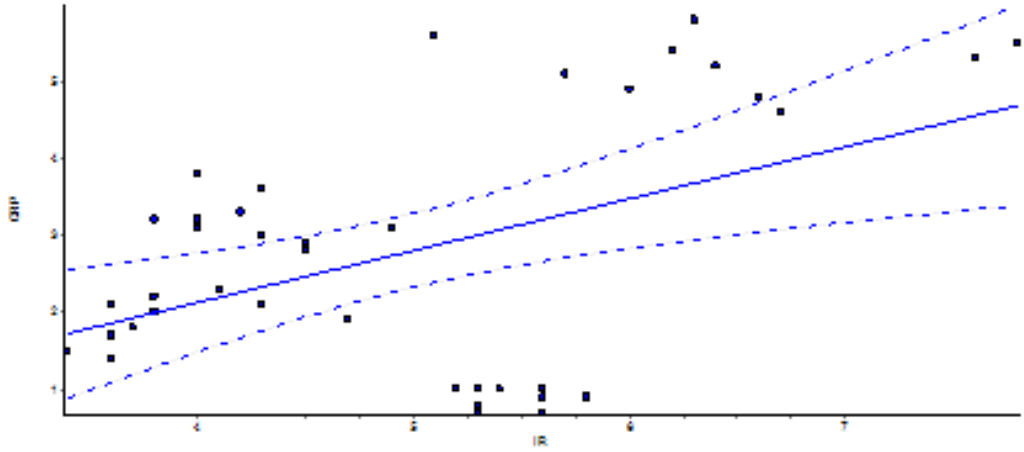
Figure (5): Correlation between Insulin Resistance (IR) and C- reactive Protein (CRP).



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