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RESEARCH ARTICLE

SILYMARIN AMELIORATES LIVER DAMAGE BY MODULATING OXIDATIVE STRESS AND APOPTOSIS IN LIVER OF STREPTOZOTOCIN-INDUCED DIABETIC RATS.

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Abstract

Objective:- Hepatic injury and cell death in diabetes is a major health problem worldwide. Oxidative stress is a contributing factor in diabetic complications including liver. Various preclinical and clinicalreports have indicated the antidiabetic effect of silymarin (SMN). The objective of this study was to investigate the effect of SMN on oxidative stress and apoptosis in liver of streptozotocin (STZ)-induced diabetes in rats.

Methods- : Streptozotocin (STZ, 50 mg/kg, ip) was injected into male rats, and after diabetic induction SMN (80mg/kg) was orally administered for 21 days.

Results:- STZ produced remarkable hyperglycemia and a decrease in insulin level. This is accompanied by increased alkaline phosphatase activity and bilirubin level with remarkable decrease in albumin content in serum of diabetic rats. A significant increase in oxidative stress was demonstrated by increased lipid peroxidation with a decrease in the glutathione levels in liver of diabetic rats. Increased percentages of apoptosis with down-regulation of Bcl-2 and activation of Bax as well as the caspases-3 and 9 were demonstrated in the diabetic rats. SMN-treated rats showed significantly lower levels of blood glucose,bilirubin and alkaline phosphatase,and higher levels of insulin and albumin when compared with diabetic group.SMN also prevented the increase in MDA and stimulated the GSH production in liver of STZ-treated rats indicating improved hepatic redox state. SMN-treated rats showed significantly higher level of Bcl-2 and lower expression levels ofBax and the caspases-3 and 9 when compared with diabetic rats. These effects might contribute and lead to control apoptosis in the liver of diabetic condition.

Conclusion:- SMN has an ability to protect liver by modulating redox stateand apoptosis.These data indicate that SMN may be useful as a therapeutic agent for liver injury in type 1 diabetes.

Introduction:-

Diabetes and its complications are associated with increased oxidative stress which triggered by increasing production of mitochondrial reactive oxygen species, by glucose autoxidation and by non-enzymatic glycation of proteins (Bojunga et al., 2004). Several reports indicate considerable changes in oxidative stress and cell death. Apoptosis, as a major result of hepatic abnormalities, is an important cause of various hepatopathies. Oxidative stress starts at early onset of diabetes mellitus and increases progressively (Kakkar et al., 1998) and can induce apoptosis in heart(Alabdan, 2015).

Although considerable improvement in the management of diabetes by synthetic drugsthe search for natural hepatoprotectiveagentsis continuously active. Medicinal plants is an important source of active natural products which differ widely due to their structure and biological properties and play an important role in the management of various human diseases, including diabetes (Saravanan et al., 2013). Many plant extracts and their purified compounds have been reported to control blood glucose and reverse physiological abnormalities(El-Abhar and Schaalan, 2014). Silymarin (SMN) is a polyphenolicflavonoid extracted from Silybummarianum L. (milk thistle) that grows in different parts of the world.SMN contains 50 - 70% of flavolignans such as silybin, silycristin, silydianin and isosilybin as the major constituents(Pepping, 1999).

SMN possesses a variety of pharmacological activities, such as anti-inflammatory,immunomodulatory, and anti-oxidant(Polyak et al., 2007, Shaker et al., 2010, Li et al., 2012). It is reported that SMN has antidiabetic activity in streptozotocin treated Type-1 DM in male rats (Huseini et al., 2006, Sheela et al., 2013). SMN reduced blood total lipids, triglycerides, total cholesterol and lipoprotein in diabetic rats (Alabdan, 2015). It alsohelp to decrease oxidation of low density lipoprotein, cholesterol biosynthesisand the development of diet induced hypercholesterolemia in rats (Krecman et al., 1998).SMN inhibits TNF-K-induced expression of adhesion molecules inhuman umbilical vein endothelial cells (Kang et al., 2003). SMN modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver (Patel et al., 2010) and prevent liver damage (Shaker et al., 2010). The mechanism of action for these beneficial effects of SMN in diabetes on liver needs further studies even though antioxidant effect is a leading theory.It is reported that, besides its free radical scavenging properties, SMN increases antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, and inhibits lipid peroxidation(Bosisio et al., 1992). The protective effect of SMN on pancreatic β-cells is reported(Matsuda et al., 2005). Notably, SMN inhibited the production of inflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α,(Schumann et al., 2003) from macrophages and/or T-lymphocytes, which mightinduce the destruction of β-cells in the development of type 1 diabetes. Recently, we showed that SMN ameliorated oxidative stress and normalized apoptotic regulating proteins and apoptosis in diabetic heartof rats (Alabdan, 2015).

Since oxidative stress induced apoptosis have been identified to be a triggering factor in diabetic complications andthere is many reports indicating the changes in parameters of oxidative stress and apoptotic proteins in diabetes mellitus, we aimed to identify the hepatoprotective activity of SMN against apoptosis and oxidative stress in experimental diabetes in male rats.

Material and Methods:-

Chemicals:-

Streptozotocin(STZ) and Silymarin (SMN) were purchased from SigmaChemical (St Louis, MO). All other chemicals are of high purity grade.

Animal and exper imental design:-

Male Wister rats weighing 180 ± 20 g, bred at the university experimental animal care center,were housed in cages with free access to food anddrinking water. The animals were kept at 22± 2 °C with a12-h light/dark cycle. All procedures fulfilled the Kingdom of Saudi Arabia Guidelines for the Use and Care of Laboratory Animals. Rats were divided randomly into four groups of 6 rats each. The first groupserved as a control, the second group received a daily oral doseof SMN (80 mg /kg) for 15 days(Sheela et al., 2013), the third groupreceived a single injection of 50 mg/kgSTZi.p,the fourth group received a single injection of STZ (50 mg/kg, i.p.) followed by SMN(80 mg/kg, i.g.)daily for 15 days.

Diabetes induction:-

To induce diabetes, the rats were injected intraperitoneally (i.p.) with freshly prepared streptozotocin in 0.01 M citrate buffer, pH 4.5, at a single dose of 50 mg/kg body wt.

Blood glucose level was determined every three days after STZ injection using a glucose monitor set (Elegance, CT-X10, Convergent Technologies GmbH & Co. KG, Marburg, Germany). The animals were considered diabetic if the blood glucose level was ≥250 mg/dl.

At the end of the experimental period, all rats weresacrificed the next day after an overnight fast.Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.)and blood was collected by cardiac puncture. Sera were separated by centrifugation (500×g) (HERMLE LABORTECHNIK Z 326K, Germany) for 5 min for biochemical

determinations.Each liversample was homogenized in 10 volumes of 50 mM sodium phosphate buffer (pH 7.4) at 4°C for 30 s using a Polytron homogenizer. The homogenate was filtered through gauze and the filtrate was centrifuged (1088×g) for 5 min in a refrigerated centrifuge. The resulting supernatant was used for the measurements of antioxidant enzymes activities.

Biochemical estimation:-

The serum glucose,total proteins, albumin, and bilirubin were estimated using kits frombio-Merieux, France. The serum insulin level was measured by ELISA method using DRG Elisa insulin kit (Rat ELISA Kit, Cambridge, MA, USA).Lipid peroxidation product, malondialdehyde (MDA), was measured by thiobarbituric acid assay, which is based on MDA reaction with thiobarbituric acid to give thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979)a red species that absorbs at 535nm (Ohkawa et al., 1979). The levels of reduced glutathione (GSH) were determined at 412 nm as previously described (Sedlak and Lindsay, 1968).

Molecular analysis:-

Flow cytometric analysis of apoptosis with Annexin V–FITC staining:-

For the annexin V assay, liver samples were stained with fluorescein isothiocyanate-conjugated annexin V using the ApoAlert kit from Clontech (Palo Alto, CA) according to the manufacturer's instructions.

Flow cytometric analysis of Bcl-2 and Bax:-

Liver samples were prepared with PBS/BSA buffer then were incubated with Anti-Bcl-2 [100/D5] antibody (ab692) or Anti-Bax [6A7] antibody (ab5714) for 15 minutes at room temperature. Cells finally was re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed by flow cytometer.

Flow cytometric analysis of caspases-3 and 9:-

Liver samples were prepared with a PBS/BSA buffer were incubated with antibody (FITC Rabbit Anti- Active Caspase-3 (CPP32; Yama; Apopain, BD Bioscience) or Rabbit monoclonalAnti-Caspase-9 (E23) antibody (ab32539) mixed well and incubated for 30 min at room temperature. The cells were washed with BD Perm/Wash (BD Bioscience), centrifuged at 400 xg for 5 min and the supernatant was discarded. Cells finally were re-suspended in BD Perm/Wash and analyzed using flow cytometer.

Statistical analysis:-

Statistical analysis of the results was performed usingANOVA followed by Newman–Keul's*post hoc* test.

Results:-

The glucose level in both normal control and SMN-treated rats showed comparable values during the experimental period. A single dose of STZ produced gradual and markedly increasing blood glucose level throughout the experimentation. On the other hand, SMN treatment significantly reduced the blood glucose level toward and close to the control values (Figure 1a).Rats treated with STZ secreted significantly less insulin than controls(Figure 1b).The treatment of diabetic rats with SMN showed significantly higher insulin level compared with that of the diabetic rats.

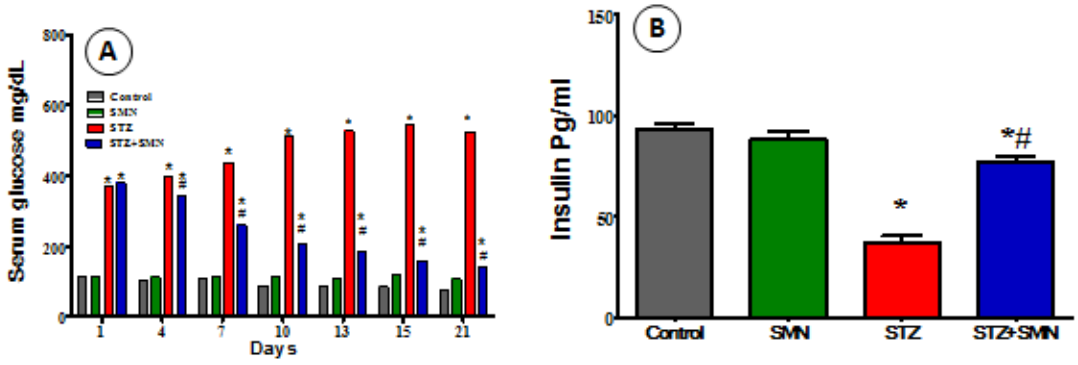

Figure 1. The effect of silymarin on the concentration of glucose (mg/dl) during the experimental period (A) and insulin (pg/ml) concentrations at the end of the experimental period of 21 days (B). All values are expressed as mean ±SEM (n=6). *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group.

The concentration of albumin in serum was significantly decreased in STZ-induced diabetes compared to the normal control animals (Table 2A). When diabetic rats were treated with SMN, the albumin level was normalized to a level comparable to that of the control rats and was significantly higher than that of the diabetic animals. Total proteins in serum was not affected by these treatments, however it showed insignificant decrease in STZ-treated rats after 21 days of diabetic induction (Table 2b). Animals that received STZ had significantly higher levels of bilirubin and alkaline phosphataseactivity when compared with control values (Figures 2C and 2D respectively). Administration of SMN for 21 days after diabetic induction ameliorated the increase in the level ofbilirubin and ALP activity in serum and displayed insignificant changes when compared with the control.

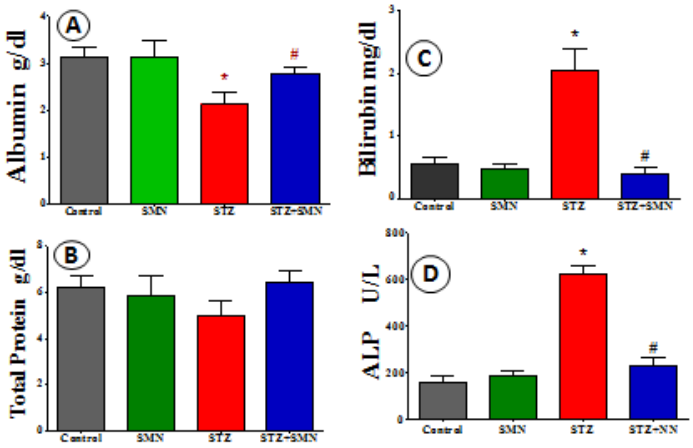

Figure 2. The effect of silymarin on the level of albumin (A), total proteins (B), bilirubin (C) (mg/dl) and alkaline phosphatase activity (U/l) (D) of experimental groups. All values are expressed as mean ±SEM (n=6). *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group.

In addition, a significant increase in lipid peroxidation (LPO) was induced inliver, as indicated from the rise of MDA concentration after STZ treatment (Figure 3A). The present results also revealed a marked amelioration of LPO in liver against diabetic-induced rise in MDA level by oral administration of SMN every other day for 21 days after STZ treatment.The concentration of GSH in liver was decreased significantly after 21 days of diabetic induction by STZ (Figure 3B). This effect was prevented significantly in liver by oral administration of SMN into diabetic rats.

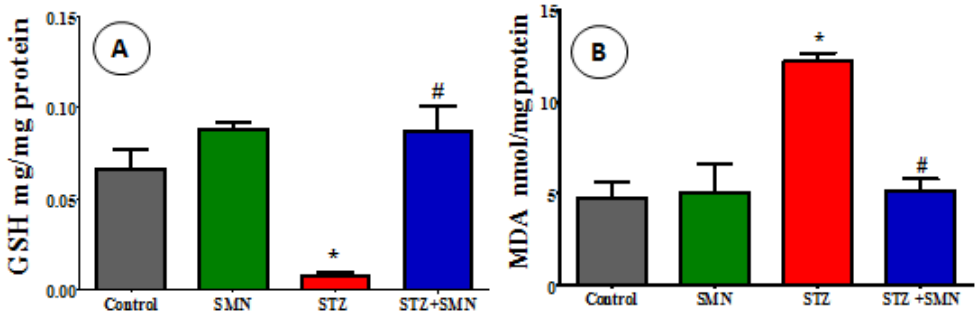

Figure 3. The effect of silymarin on lipid peroxidation product (MDA, nmol/mg protein) (A) and glutathione (GSH, mg/g protein) (B) levels in liver of experimental groups. All values are expressed as mean ±SEM (n=6). *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group.

Flow cytometry was used to assess apoptosis in liver using Annexin V-FITC staining (Figure 4). The percentages of apoptotic cells were significantly higher in livers of the diabetic rats than in those of turehe controls. The SMN therapy considerably minimized the number of apoptotic cells compared to the diabetic rats.

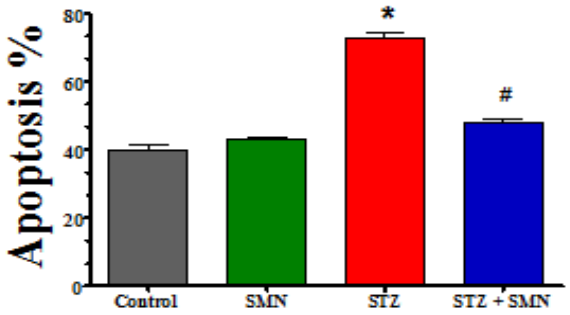

Figure 4. Flow cytometry analysis of rat liver showing the percentage of apoptosis in different animal groups. All values are expressed as mean ±SEM (n=6). *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group.

The analysis of the flow cytometry data revealed that the anti-apoptotic protein Bcl-2 was significantly reduced, while the apoptotic protein Bax was augmented in livers of STZ-induced diabetic rats (Figures 5A & 5B). SMN treatment considerably normalized the STZ-induced changes in the expression of these proteins. The expression levels of caspases-3 and 9 were significantly upregulated in livers of the STZ-induced diabetic rats. SMN administration significantly normalized these apoptotic proteins (Figures 5C & 5D).

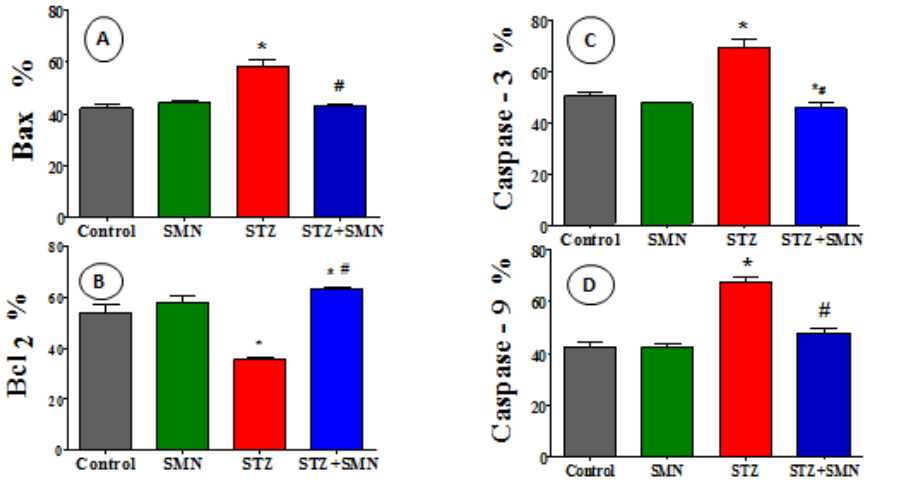

Figure 5. Flow cytometry analysis of rat liver showing the percentage of Bcl-2, Bax and caspases-3 & 9 in different animal groups. All values are expressed as mean ±SEM (n=6). *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group.

Discussion:-

The results of our study demonstrated thatthe blood glucose of diabetic rats showed remarkable continuous increase, that significantlyprevented when SMN given to diabetic rats. The present results are consistent with other reports(Shirwaikar et al., 2004, Huseini et al., 2006, Sheela et al., 2013) that SMN showed a significant decrease in the average fasting blood glucose level in drug-treated animals when compared to a diabetic group. The present results also showed that SMN prevented the decrease in insulin level in the diabetic rats that treated with SMN.SMN induces recovery of pancreatic function after alloxan damage in rats (Soto et al., 2004). These results were consistent with recent results that there was a significant decrease in glycosylated hemoglobin levels in the SMN-treated group when compared to the diabetic group (Sheela et al., 2013). In rats, SMN administration decreased serum insulin level, HOMA-IR and markedly improved myocardial damage(Alabdan, 2015). These results are also consistent with other reports thatsilybin administration decreased insulin resistanceand serum ALT activity and markedly improved hepatic and myocardial damage (Loguercio et al., 2012). The present results also showed significant amelioration of the loss of albumin and the rise in bilirubin levels and alkaline phosphatase activity in

serum of the diabetic rats that received SMN. These results suggest that SMN treatment to diabetic rats actively protected liver and ameliorated hepatic functions.

Reactive oxygen species are involved in the pathophysiology of various diseases including diabetes. Liver is an important organ which responsible for various metabolic functions. During diabetes persistent hyperglycemia causes generation of ROS that exert hepatotoxic effect by peroxidation of membrane phospholipids leading to changes in permeability and loss on membrane integrity ultimately lead to cell death. The present study demonstrated a significant elevation of lipid peroxidation product and a decrease in GSH levels inliver. The results clearly indicateincreased production of free radicals in STZ-induced diabetes leading oxidative injury of liver cells. In group treated with SMN, the elevated oxidative stress was ameliorated in liver.Thus, SMN helped to scavengeROS and the lipid peroxidation products produced excessively by diabetes leading to protection of the liver. Similar study has established that SMN treatment prevented tissue damage and restored the activity and gene expression of antioxidant enzymes after alloxan administration (Soto et al., 2010).It has been shown that SMN prevents the damage induced by oxidative agents in hepatic membranes, microsomes and mitochondria (Wu et al., 2003, Turgut et al., 2008). SMN has been approved as a safe herbal product for renal protection in high doses. It has antioxidant effects via increase of gene expression of antioxidant enzymes and a number of the most important protection mechanisms against free radicals damage containing superoxide dismutase, glutathione peroxidase, and catalase(Sheela et al., 2013).These observations of the effect of SMN of hepatocyte protection may contribute to explain why this compound has a protective effect on lipid peroxidation in liver

It has been reported that reducing sugars trigger oxidative modification and apoptosis in pancreatic cells by provoking oxidative stress through glycation reaction (Kaneto et al., 1996). However, information on the occurrence of oxidative stress in liver at early stages of development of diabetes is lacking. The present study demonstrated altered redox state in the liver and a significant increase in the expression of apoptotic protein Bax and a decrease in the anti-apoptotic protein Bcl2 with activation of caspase-3, and 9 supporting the increase in the number of apoptotic cells in the diabetic group. The disruption of these proteins indicates apoptosis developing through the integral pathway of apoptosis (Khurana et al., 2014). Because of the antioxidant potential of SMN it is anticipated that death and survival of hepatocytescan be regulated by antioxidant and anti-apoptotic proteins.In this study, anti-apoptotic expression of Bcl2 and blocking Bax and caspases-3 & 9 were remarkable in liver of the SMN-treated diabetic group, but that was not the case for those in the diabetic group indicating a control of apoptosis through mitochondrial pathway. Control of glycaemia and oxidative stress with lowered generation of ROS in cells treated with SMN could prevent apoptotic signaling in the mitochondria through specific targeting of Bcl2/caspase-3 and improved mitochondrial membrane potential and membrane fluidity (Zhu et al., 2014). Moreover, SMN may prevent the activation of caspase-3 by controlling its upstream mitochondrial cytochrome c pathway (Zhu et al., 2014). In SMN-treated diabetic rats, the down-regulation of the caspases and up-regulation of Bcl-2 indicated that SMN directly blocks main steps in the mitochondrial apoptotic pathways. Thus, it appears that SMN maintained the amount of ROS generation and free radical detoxification systems within the levels required for optimal anti-apoptotic processes. This important balance has crucial effects on the control of redox-sensitive signaling proteins and apoptotic signals through the redox-sensitive pathways of apoptosis.

Conclusion:-

The protective effect of silymarin on liver of diabetic rats is demonstrated. Importantly, silymarin has an ability to prevent the loss of albumin, the rise of ALP activity and bilirubin level in diabetic rats. It also prevented the decrease of glutathione and the development of lipidperoxidarionwhich probably contribute in protecting liver integrity. Silymarin also has an ability to inhibit the activation of apoptotic signaling proteins, such as Bax,and caspases-3 and 9 as well as activate antiapoptotic protein such as Bcl-2 in liver cells, which probably control the progression of apoptosis in liver.Taken together, these data indicate that silymarin maybe useful as a therapeutic agent for liver injuryin type 1 diabetes.

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