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THE EVALUATION OF CANCER INCIDENCE SUSCEPTIBILITY IN THE LIVER OF TYPE 1 AND 2 DIABETIC RATS

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ABSTRACT

The relationship between diabetes and cancer development is not clear. But the risk to develop cancer is increased if the person is diabetic. In the present study we induced type 1 and type 2 diabetes in rats by streptozotocin and high fat diet, respectively. Many cell growth and apoptosis related antigens were studied including tumour suppressor antigen; P53, anti-apoptotic protein bcl-2, pro-apoptotic protein bax, cyp1a2 and cyp2e1 and caspase 3. In type 1 and type 2 diabetes cyp2e1, bax, p53 and active caspase 3 were upregulated but active caspase 3 was prominent active in type 1 compared to type 2 diabetes. Both cyp1a2 and bcl-2 were down regulated in both types of diabetes. Bcl-2 was dramatically decreased in type 1 than type 2 diabetes. Normal or high insulin dose treatment could restore the basal line of most parameters but partially restored the level of cyp1a2 and could not restore the level of p53 in type 1 diabetes. High P53 level in both types of diabetes indicates cellular stress and damage. The high level of P53 was accompanied with active caspas3 and reduced bcl-2 in type 1 but not type 2 diabetes.

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INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects of insulin secretion and/or increased cellular resistance to insulin. Type1isinsulin-dependent diabetes mellitus (IDDM), this form of diabetes represents for only 5-10% of diabetics. IDDM isan autoimmune disease in which the immune system destroys the insulin-making beta cells of the pancreas (Betterle *et al*., 1984). Non-insulin-dependent diabetes mellitus (NIDDM), referred to as type2 diabetes. The majority of patients with this form of diabetes are obese, or have an increased percentage of body fat distributed mostly in the abdominal region resulted in insulin resistance (Kissebah *et al*., 1982; Campbell and Carlson, 1993). Cancer is a group of diseases which characterized by insufficient negative regulation of cell growth. In addition to evasion of cell death, DNA damage and oxidative, mitotic, proteotoxic and metabolic stresses, are also characters of cancer (Hanahan, and Weinberg, 2000; Luo, *et al*., 2009).

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Diabetes and cancer represent two complexes, chronic and possibly fatal diseases. There is a growing body of evidence published in recent years that suggest significant increase in cancer incidence in diabetic patients. It was stated that 8–18% of cancer patients have diabetes (Habib and Rojna, 2013). The worldwide prevalence of diabetes was estimated to rise from 171 million in the year 2000 to 366 million in the year 2030, that may cause the possibility of raising cancer patients as well. Tumor related antigens levels as Bcl-2, CYP 2E1 and CYP 1A2 and cell death/survive related antigens as Bax, P53 and caspase-3 will give good materials for studying the relationship between diabetes and cancer. The caspase family is the largest enzymes involved in apoptosis. They synthesized as proenzymes, and appear distributed in many locations including cytoplasm, nuclear matrix or mitochondrial inter membrane space (Chandra *et al*., 2001). P53 is a tumor suppressor that is found in modified or functionally inactivated form in human cancers. P53 is activated in response to DNA damage, hypoxia, expression of certain oncogenes and many cytotoxic stimuli. The p53 functions prevent tumor progression by inhibiting the proliferation of damaged or stressed cells through activation of apoptosis, autophagy or senescence (Michalak *et al*., 2005; Zhivotovsky and Orrenius, 2010).

Bax (Bcl-2-associated X protein) found in mitochondrial membrane and on the inner nuclear membrane but not present on other organelles such as the endoplasmic reticulum (Henshall *et al*., 2002). Bax is a pro-apoptotic considered important factor in the initiation of cell death pathways (Korsmeyer *et al.,* 2000; von Ahsen *et al*., 2000) through conformational change and localization to mitochondrial membranes (Gross *et al*., 1998; Nechushtan *et al*., 1999). By this way, it promotes release of cytochromec, the latter can promote caspase activation and initiate apoptosis (Matsuyama and Reed, 2000). Bcl-2 (B-cell lymphoma/leukemia-2 gene) Participates in B-cell malignancies (Tsujimoto *et al*., 1985). It found on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope (Krajewski, *et al*., 1993). Bcl-2 prevents programmed cell death through maintaining mitochondrial integrity (Yang *et al*., 1997; Stanley and Korsmeyer, 1999). The cytochrome P-450 (CYP) represents a super family of hemoproteins that mediate the biotransformation of endogenous and exogenous compounds.

The isoform E1 of the CYP2 subfamily (CYP2E1) is the only member in humans, rats and mice that stimulate the bioactivation of several procarcinogens and involved in drug metabolism (Caro and Cederbaum, 2004; Abdelmegeed, *et al*., 2005). CYP2E1 has a wide tissue distribution, being highly expressed in the liver but also in many other cell types (Saito, *et al*., 1997; Yoshinari *et al*., 2004). CYP1A2 is a constitutive enzyme that is expressed mainly in the liver. It constitutes approximately 13 % of the total hepatic CYP content. This enzyme catalyzes the metabolism of a wide range of chemicals; several of them are suspected human and/or animal carcinogens (Shimada *et al*., 1994; Hakkola *et al*., 1998). Taking into consideration the above mentioned information, the main objectives of the current study is to explore the relationship of diabetes and cancer. This will be achieved by studying the levels of different cell death and growth proteins as P53, CYP 2E1, CYP1A2, Bax, Bcl-2 and caspase 3 in liver tissue of two different diabetic rat models. Streptozotocin (type 1) induced diabetes and in high fat diet induced diabetes (type 2).

MATERIALS AND METHODS

Materials

Tween 20, EDTA (ethylene diamine Tetra-acetic acid), EGTA (ethylene glycol Tetra-acetic acid), Sodium dodecylesulphate polyacrylamide gel electrophoresis (SDS-PAGE) chemicals, Nitrocellulose membrane, protease inhibitor cocktail, mouse anti-P53 IgG, mouse anti bcl2 IgG, mouse anti bax IgG, mouse anti cyp 2e1 IgG and mouse anti cyp1a2 IgG were purchased from Sigma (USA). Super Signal West Pico Chemiluminescent Substrate was from Pierce Biotechnology (USA). Mouse anti-caspase 3 IgG, goat anti-mouse IgG-HRP, goat anti-β actin IgG and mouse anti-goat IgG-HRP are from Santa Cruz Biotechnology (USA). Protein Assay Kit was from Bio-Rad (Austria). All other chemicals of good quality -if not mentioned- were obtained from local commercial sources.

Experimental design and animals

Fifty adult male rats weighing 250-300 g obtained from King Khalid University animal house. Suitable temperature of 20 ± 4 C° and lighting cycle of 12 hours light /dark were also into consideration. All animals were given free access to standard chow and healthy water. All experimental procedures were conducted in strict compliance with the guide of National Institute of Health for the Care and Use of Laboratory Animals. Animal were categorized into 5 groups, each of 10 rats as follow. Control group (cnt) served as normal healthy group. Streptozotocin group (stz) received Streptozotocin 50 mg/Kg one IP injection every day for 5 days and left for 20 days after induction of diabetes. Streptozotocin and normal insulin group (stz +ins) will receive Streptozotocin as group 2 and then 0.75 U/Kg recombinant human insulin for 20 days after induction of diabetes. Streptozotocin and high insulin group (stz +hins) will receive Streptozotocin as group 2 and then 1.3U/Kg recombinant human insulin for 20 days after induction of diabetes. High fat diet group (hfd) will receive 5% of glucose in free access drinking water with high fat rodent chow (46% energy from fat).

Fasting Blood glucose and insulin levels measurements

Rats were fasted overnight and then glucose level was measured from whole tail vein blood with a hand-held glucose test monitor (Lifescan, Johnson and Johnson) and expressed as mg/dl. Serum insulin was quantified using rat insulin ELISA kit (Crystal Chem, USA) according to manufacturer instructions.

Western blot

Liver tissue was lysed in RIPA and grinded by electronic blender for 15 sec. Centrifuge for 20 min (12000 rpm, 4°C).Protein concentration of the supernatant was estimated. Equal protein concentration of each group was loaded with sample buffer and boiled for 5 min to denaturation, then stored at -80 °C until use. After SDS-PAGE, transfer of protein bands into nitrocellulose membrane was done by dry blot, blocking of active sites was carried out for 2 hours using 5% skim milk in 1 % TBST. Detection of different tumor related and cell death antigens (P53, P21, P450, Bax and Bcl-2) were carried out by using proper antibodies for each antigen according to manufacturer instructions. Incubation with first Ab overnight was done in closed plastic pages in 1% TBST at 4 °C. Secondary Ab HRP conjugated was applied for one hour at room temperature in the same buffer as first Ab. Detection of the specific antigen was carried out by ECL kit. The optical density of each protein was carried out by Image J software and normalized to the corresponding actin band.

Statistical analysis

All data were presented as mean ± SD. Statistical analyses were performed by using an ANOVA. P value less than 0.05 was considered significant.

RESULTS

Fasting blood glucose and insulin levels

The fasting blood glucose level (fbgl) showed significant increase instz and hfd groups when compared with cnt group (Fig 1A). In contrast, it decreased significantly in stz+ins and

stz+hins groups. This result indicates the incidence of hyperglycemia of stz and hfd groups. Serum insulin was reduced in stz group and restored more or less near the control level in stz+ins (Fig 1B). High insulin treatment caused high serum insulin level, but the highest serum insulin level was obtained from hfd group. The status of serum glucose and insulin levels was summarized in Table 1. The different states of glycemia and insulinemia obtained in this investigation may enable to determine which status is more susceptible for cancer development.

Modulation of CYP 450 in diabetic liver

The detection of two members of CYP 450 belonging to two subfamilies namely; cyp2e1and cyp1a2 was carried out in different treatments. cyp2e1 (Fig 2A) increased in stz and hfd groups compared to the cnt group, as indicated by densitometric measurements (Fig 2B). The level of cyp2e1 is more or less returned to the basal line of cnt after treatment with insulin in stz+ins and stz+hins groups.CYP1a2 is shown in (Fig 2A).

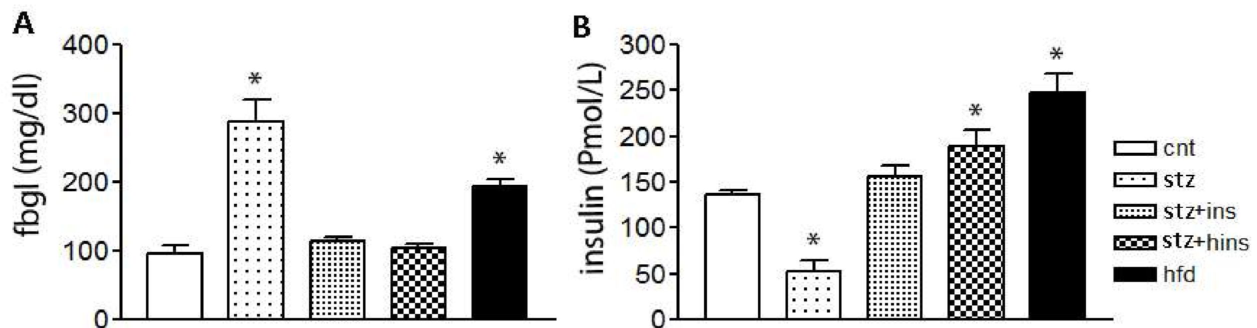

Fig. 1. Fasting blood glucose and insulin levels in different treatments: fasting blood glucose (fbgl) (A) and insulin levels (B) were estimated as described in method section, data representing the mean ±SD, * P < 0.05 compared with control

Table 1. Status of glucose and insulin levels in different groups of the experiment

Experimental group	Description
cnt	Normoglycemic and Normoinsulinemic
stz	Hyperglycemic and Hypoinsulinemic
stz + ins	Normoglycemic and Normoinsulinemic
stz +hins	Normoglycemic and Hyperinsulinemic
hfd	Hyperglycemic and Hyperinsulinemic

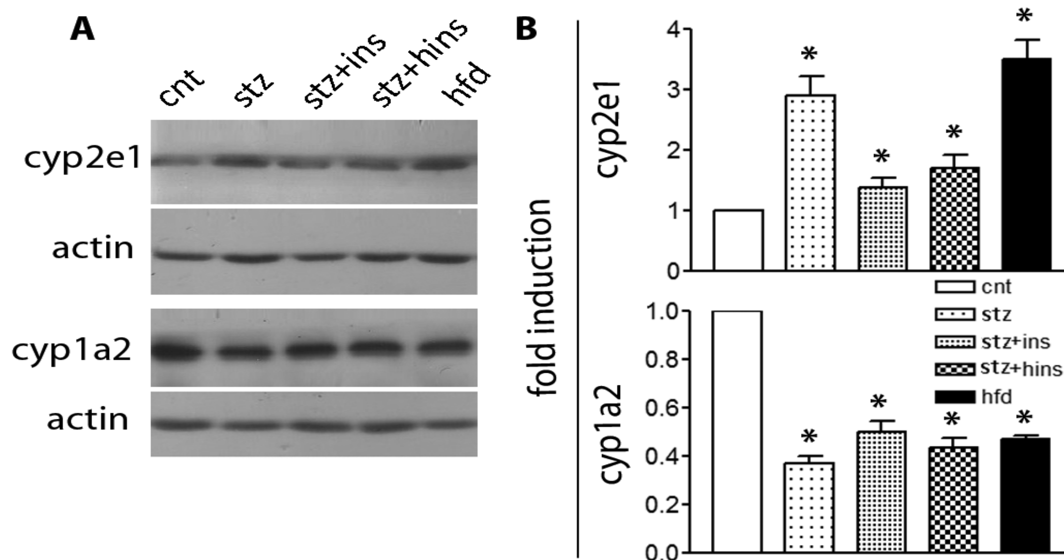

Fig. 2. Immunodetection of cyp450 subtypes: cyp2e1 and cyp 1a2 were detected by western blot in liver tissues of different treatments (A). Estimation of band optical density was carried out by Image J software and normalized to the corresponding actin band and showing as fold induction (B). Here is a representative figure of at least three independent experiments. Data representing the mean ±SD, * P < 0.05 compared with control

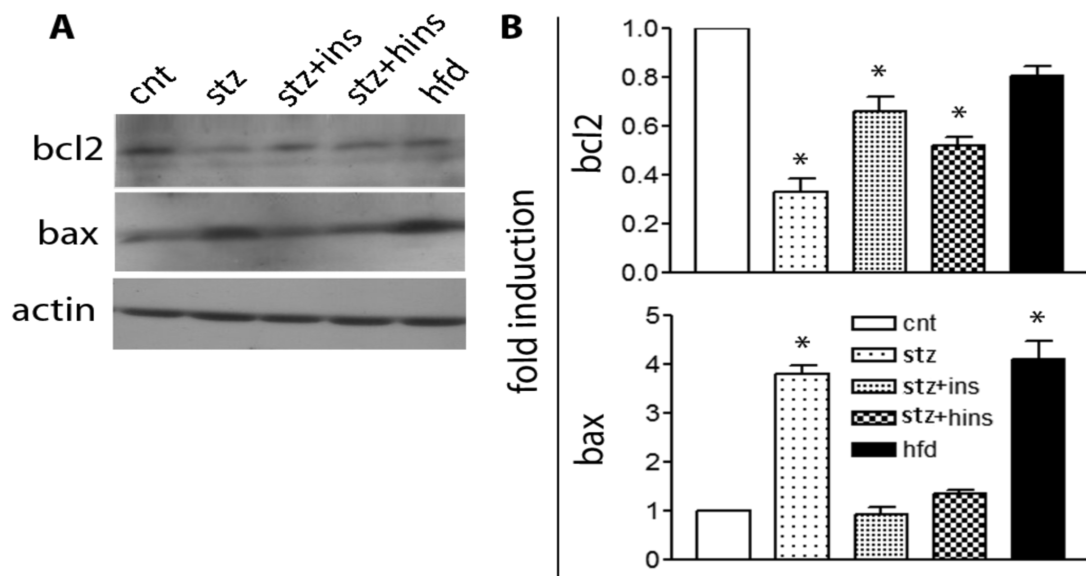

Fig. 3. Immunodetection of bcl2 and bax: Bcl2 and bax proteins were detected by western blot in liver tissues of different treatments (A). Estimation of band optical density was carried out by Image J software and normalized to the corresponding actin band and showing as fold induction (B). Here is a representative figure of at least three independent experiments. Data are representing the mean ±SD, * P < 0.05 compared with control

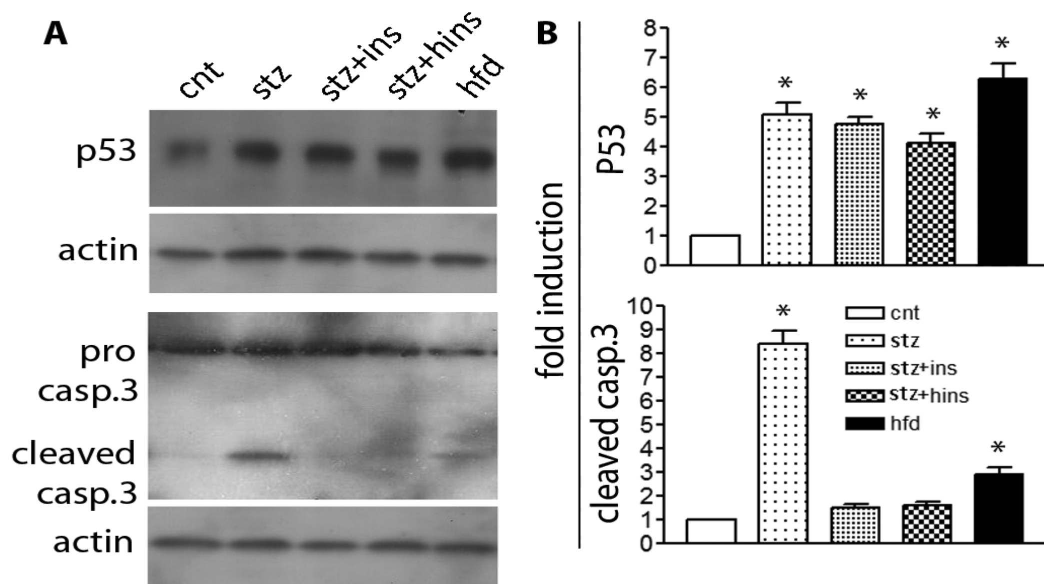

Fig. 4. Immunodetection of P53 and caspase 3: P53 and active caspase 3 were detected by western blot in liver tissues of different treatments (A). Estimation of band optical density was carried out by Image J software and normalized to the corresponding actin band and showing as fold induction (B). Here is a representative figure of at least three independent experiments.Data representing the mean ±SD, * P < 0.05 compared with control

The level of cyp1a2 decreased in stz, stz+ins, stz+hins and hfd groups compare with cnt group as indicated by densitometric measurements (Fig2B). Notably, the level of cyp1a2 increased little instz+ins when compare with stz group.

Incidence of diabetes modulates bcl-2 and bax proteins in liver

The level of the anti-apoptotic protein bcl-2 decreased sharply in stz group and to a little extent in hfd group (Fig 3 A).Partial restoration of bcl-2 level obtained in stz+ins and stz+hins groups (Fig 3 B).

In contrast, the pro-apoptotic protein bax shown in (Fig 3 A) dramatically increased in stz and hfd groups compared to the cnt group. There is no significant restoration of bax level instz+ins and stz+hins groups (Fig 3 B).

High expression of P53 and induction of apoptosis in diabetic liver

The tumor suppressor antigens P53 reached the highest level in hfd and stz groups (Fig 4 A). Insulin treatment in both normal and high doses did not antagonize the action of diabetes (Fig 4 B). It is well known that P53 may induce

apoptotic cell death under certain cell stress. Accordingly we aimed to measure the activation of the executioner caspase 3. Immunoblot detection of cleaved caspase3 (Fig 4 A) revealed obvious increase in stz group compared with cnt (Fig 4 B). The level of cleaved caspase3 was slightly increased in hfd than cnt group. Treatment with insulin in stz+ins and szt+hins groups resulted in reduction of cleaved caspase3 back to the basal line of cnt.

DISCUSSION

Most of insulin-producing β cells are degenerated or had necrosis uponstreptozotocin treatment leading to a decrease in insulin secretion and an increase in blood glucose concentration (Adeyemi *et al*., 2010). Intracellular action of stz may be according to DNA fragmentation (Morgan *et al*., 1994) or alkylation (Delaney *et al*., 1995; Elsner *et al*., 2000) in pancreatic β cells. In this study, the injection of recombinant human insulin subcutaneously (0.75 U/kg) in stz+ins group and (1.3U/Kg) in stz+hins group daily for 20 days cause decrease in fasting blood glucose levels of stz group. In this case, external insulin use as substitute to original insulin that secreted by β cell after destroyed by stz, this caused significant decrease in blood glucose level. This result is in line with other studies, (Nordquist and Sjöquist, 2009). Significant increase of fasting blood glucose level and high serum insulin was obtained by the administration of oral glucose together with high fat feeding. Feeding of rats on hfd for two weeks was enough to produce insulin resistance syndrome characterized by the increased body weight (obesity), mild hyperglycemia, hypertriglyceridemia, hypercholesterolemia and compensatory hyperinsulinemia (Reaven, 1991). Hyperglycemia resulting from insufficient levels of insulin or its action on cells causes free radicals production which leads to oxidative stress and apoptosis in various cells (Arya, *et al*., 2011). Apoptosis is regulated by several proteins, the most important one is p53 which normally activated by cellular stress and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis (Kuribayashi and El-Deiry, 2007).

In addition, p53 reduces the expression of anti-apoptotic genes such as bcl2 and up regulates genes promoting apoptosis such as bax (Leri, *et al*., 1998). Both bcl2 and bax play a main role in regulating apoptosis (Nogueira, *et al*., 2009). It has been shown that bax can promote caspase activation and initiate apoptosis (Matsuyama and Reed, 2000). There areanti-apoptotic proteins which may be affected by the oxidative stress which produced from hyperglycemia as cyp2e1, cyp1a2 and bcl2. In fact, the increase in cyp2e1 level was previously reported in stz- induced diabetes in rats (Yao *et al*., 2009; Saravanan and Ponmurugan, 2013) and in hfd-induced insulin resistance (Mantena, *et al*., 2009). In general, the expression of cyp2e1 is induced by many of endogenous and exogenous compounds including ethanol, acetone, and other low-molecular-weight substrates and by hfd and insulin-deficient diabetes (Schattenberg, *et al*., 2004; Abdelmegeed, *et al*., 2005). Low level of cyp1a2 (Pass, *et al*., 2002) and bcl2 (Hasnan, *et al*., 2010; Kapoor and Kakkar, 2014) in diabetic animals was recorded. High level of bax in diabeticanimalsis logic as the reduction of bcl2 level. It indicates the incidence of apoptotic cell death in hyperglycemia.

The up regulation of bax level was also reported in diabetes (Kapoor and Kakkar, 2014) and after feeding with hfd (Wang, *et al*., 2008). In the current study we observed high level of p53 in stz and hfd groups. It was reported that P53 activation in type 2 diabetes was because of the increase in oxidative stress levels (Goldstein and Rotter, 2012; Yahagi, *et al*., 2004). We cannot exclude the action of oxidative stress in our study. Furthermore, high glucose-induced oxidative stress causes DNA damage and can stimulate apoptosis via p53-related mechanisms (Allen, *et al*., 2005). High level of active caspase 3 in the present investigation lined with (Wang, *et al*., 2008; Kapoor and Kakkar, 2014), they showed increased gene expression of caspase-3 in stz and hfd groups respectively. Our results suggest that the studied cell death and growth antigens were all modified in insulin dependent and independent diabetes. This may increase the possibility of cancer development potential in diabetes.

Conclusion

Both type I and 2 diabetes induces liver damage and modulation of tumor related and apoptotic antigens. More cellular damaged have been occurred in type 1 than type 2 diabetes. Insulin treatment partially rescued these damages. So seeking for modern regimens to controlling blood insulin level in accordance with blood glucose level may be the best solution for avoiding diabetic complications.

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