Scholars LITERATURE

La Prensa Medica Argentina

Research Article

DOI: https://doi.org/10.47275/0032-745X-264
Volume 106 Issue 6

The Effect of Diabetes on the Histological Parameters in Alloxan Induced Diabetes Male Rats

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Abstract

This study aimed to determine the effect of high blood glucose on the histological variables in alloxan-induced diabetes male rats. Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from insufficiency in the production or action of insulin produced by the pancreas inside the body. Sidewise to hyperglycemia, several other factors play a great role in the pathogenesis of diabetes such as hyperlipidemia and oxidative stress leading to a high risk of complications. Alloxan, which is chemically known as 5,5-dihydroxyl pyrimidine-2,4,6-trione is an organic compound, is the most widely used in diabetes studies. During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS) for all tissues from glucose auto-oxidation and protein glycosylation. Free radicals may play a crucial role in the causation and complications of diabetes mellitus. The induced diabetes led to additional changes in the liver tissues of infected animals (G2,G3) compared with the control group (G1), as shown in the liver of group 2 there is a dilatation of portal vessels with prevascular inflammatory cell infiltration and dilatation of central vein congestion with inflammatory cell aggregations while we show in the group 3 severe congestion and dilatation of central vein with vacuolated hepatocytes and atrophy of sinusoid, marked coagulative necrosis (nuclei with karyolysis and karyorrhexis) with inflammatory cell proliferation and binucleate hepatocyte and hepatocellular atrophy and correspondingly wide sinusoids with clear pyknotic nuclei of apoptotic cells. Hepatocytes showing different stages of mitotic divisions. The induced diabetes led to a significant increase in apoptotic cell death of the liver in the diabetic groups (G2,G3) Which is expressed by the percentage (15,71%, 27,73%) compared with the control group (G1) 9.32%.

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Citation: Mukhlif ZF, Rahim SM, Jumaa Jamal MN (2020) The Effect of Diabetes on the Histological Parameters in Alloxan Induced Diabetes Male Rats. Prensa Med Argent, Volume 106:6. 264. DOI: https://doi.org/10.47275/0032-745X-264.

Received: May 03, 2020; **Accepted:** May 18, 2020; **Published:** May 23, 2020

Introduction

Diabetes mellitus is a group of diseases characterized by an elevated blood glucose level (hyperglycemia) resulting from defects in insulin secretion, or insulin action, or both [1-3]. Common symptoms of diabetes are lethargy from marked hyperglycemia, polyuria, polydipsia, weight loss, blurred vision, and susceptibility to certain infections. Diabetes mellitus can be classified in different ways, however, diabetes is mostly classified basically into two major types: Type I Diabetes (IDDM) and Type II Diabetes (NIDDM). In type I Diabetes (IDDM) insulin secretion is deficient due to the autoimmune destruction of beta-pancreatic cells (β cells) that leads to metabolic disturbances associated with IDDM [4]. The end-stage of β-cells destruction represents the onset of clinical disease leading to type I diabetes mellitus [5]. Autoimmunity, genetic makeup, and environmental factors are responsible for islets cell destruction [6]. In Type II Diabetes (NIDDM) there are some disorders in mechanisms that keep regulation between tissue sensitivity to insulin, which consequently leads to impaired insulin secretion by the pancreatic β cells and impaired insulin action through insulin resistance [7]. In this type of diabetes, multiple genetic defects, and certain environmental factors especially obesity are responsible for β cell defects and peripheral tissue insulin resistance respectively [8].

Chronic hyperglycemia causes long-term damage, dysfunction and failures of various cells, tissues, and organs. Diabetic patients are at risk of the macro and micro vascular complications through the body [9,10]. The micro vascular complications, include nephropathy, retinopathy, neuropathy, and macrovascular complications including heart disease and stroke. DM is associated with a markedly increased mortality rate from cardiovascular and renal diseases [11]. In both T1DM and T2DM, diabetic complications in target organs arise from chronic elevations of glucose. Alloxan, which is chemically known as 5, 5-dihydroxyl pyrimidine- 2,4,6-trione is an organic compound, is the most widely used in diabetes studies. Alloxan has been used to induce experimental diabetes due to the selective destruction of the insulin producing pancreatic beta-islets. Alloxan induces a multiphasic blood glucose response when injected into to an experimental animal, which is accompanied by corresponding inverse changes in the plasma insulin concentration followed by sequential ultra-structural beta cell changes ultimately leading to necrotic cell death [12]. The pathogenic effect of high glucose, possibly in concert with fatty acids, is mediated to a significant extent via increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and subsequent oxidative stress. These ROS and RNS directly oxidize and damage DNA, proteins, and lipids and tissue [13]. This generation of oxygen

free radicals during cellular metabolism, and by certain environmental factors, including lifestyle, appears to play a critical role in the pathogenesis of diabetes mellitus [14]. Reactive oxygen species have been implicated in the pathogenesis of a variety of diseases including diabetes mellitus. These diseases are classified according to the origin of ROS production into two categories, oxidative stress and inflammatory oxidative conditions. The first category includes cancer and diabetes mellitus, and is caused by the increase of ROS production in the mitochondria, damaging the cellular components such as membrane lipids, nucleic acids and proteins. The second category includes chronic inflammatory diseases in which ROS secretion is regulated by cytokines and other factors secreted by vascular and immune cells [15]. The liver is a central regulator of glucose homeostasis and stores or releases glucose according to metabolic demands [16]. In insulin resistant states or diabetes, the deregulation of hepatic glucose release contributes significantly to the pathophysiology of these conditions. Acute or chronic liver disease can aggravate insulin resistance and the physiological effects of insulin on hepatocytes are disturbed. Insulin resistance has also been recognized as an independent risk factor for the development of liver injury. In the healthy liver tissue homeostasis is achieved through cell turn over by apoptosis and deregulation of the physiological process resulting in too much or too little cell death can have potentially devastating effects on liver tissue. The delineation of the signaling pathways that mediate apoptosis changed the paradigms of understanding of many liver diseases. These signaling events include cell surface based receptor ligand systems and intracellular signaling pathways

Materials and Methods

Animals and Housing

The present study has been conducted in the animal house of the college of veterinary medicine, AL-Anbar University during the period extended from October, 2017 to April, 2018.

Fourty five mature male Wistar rats (aged 13 weeks and weighted 150±10 g) were used in the three experimental periods of the present study. Male rats were allowed to acclimatize to the animal house environment before beginning of the experiment. Animals were housed in polypropylene cages inside a well-ventilated room. Each cage consist of not more than five rats. Male rats were fed on the drinking water and ad libitum with standard rat feeds. It consists of amino acid supplemented casein, cornstarch, maltodextrose or sucrose, and soybean oil or lard, also supplemented with minerals and vitamins throughout the experiment [17]. Room temperature was maintained at 23±2ºC, the light-dark cycle was on a 12 hr light/dark cycle with light on at 06:00 a.m. and off at 06:00 p.m. during the experimental periods. The rats had oral dose 0.5 mg (Sodium Sulfadimidine) in 1 liter of water for 5 consecutive days, and 0.5 mg of Ampicillin W.S.P. (20%) in 1 liter of water for 5 consecutive days to ensure that it is free of various diseases and left the animals to acclimate for two weeks.

Experimental Design

Forty five experimental animals were obtained and divided into 3 groups with 15animals in each group as follows:

- Control group (G1): 15 male rat gavage distilled water.
- Group 2 (G2): 15 male rats induce diabetes by injection intraperitoneally with Alloxan 150 mg/kg bw for 14 days.
- Group 3 (G3): 15 male rats induce diabetes by injection intraperitoneally with Alloxan 150 mg/kg bw for 24 days.

Preparation of Alloxan Solution

To induce diabetes in rats, alloxan was used which was procured from M/s Sigma Chemicals, St. Louis, USA. Fresh 0.1 M citrate buffer of pH 4.5 was prepared and maintained at 4-8°C. Alloxan was dissolved in cold citrate buffer.

Experimental Induction of Diabetes

The animals were fasted overnight and diabetes was induced in animals of Group II and III by a single intraperitoneal injection of freshly prepared solution of alloxan at the dose rate of 150 mg/kg b.w. in 0.1 M cold citrate buffer of pH 4.5. Control group animals received distilled water alone.

After the first day, a 5% glucose solution with drinking water was given to prevent severe hypoglycemia caused by beta cells pancreatic damage and then animals were allowed to eat the feed after the injection.

Confirmation of Diabetes

The blood glucose levels were estimated 72 hrs. post alloxan injection using Glucochek glucometer (Aspen Diagnostic (P) Ltd.) The glucometer has been designed to measure the blood glucose level by GOD-POD method (Glucose oxidase-peroxidase method). The animals with blood glucose levels above 300 mg/dl were considered as diabetic.

Collection of the Samples

Male rats have been monitored throughout the experimental periods. At the end of each treated and control subgroup period, male rats were anaesthetized (by injection of 0.3ml ketamine + 0.1 ml of xylazine/ kg b.w. ip). Liver samples were placed in dry, clean plastic containers after marking them and it is fixed with formalin 10% more than 24 hrs. for the histological section preparations.

Histological Preparations

Histological examination was done after fixing the organ of rat in 10% formalin, processed and embedded in paraffin wax. Tissue blocks were sectioned 5 lm thick and stained with Harris haematoxylin and eosin (H&E) [18].

Determination of Apoptotic Index

In situ direct DNA fragmentation Assay (TUNEL-based detection Kit):

Principle of the procedure: The reagents provided by abcam/ UK with kit code abcam 14085, Abcam's Annexin V-FITC. Apoptosis Detection Kit is based on the observation that soon after initiating apoptosis, cells translocate the membrane phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. The one-step staining procedure takes only 10 minutes. Detection can be analyzed by flow cytometry or by fluorescence microscopy. The kit can differentiate apoptosis *vs.* necrosis when performing both Annexin V-FITC and PI staining.

Assay Protocol:

Incubation of cells with Annexin V-FITC

- Induce apoptosis by desired method.
- Collect 1-5 x 105 cells by centrifugation.

- Re-suspend cells in 500 µl of 1X Binding Buffer.
- Add 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI 50µg/ml, optional).
- Incubate at room temperature for 5 min in the dark. Proceed to step 2 or 3 below depending on method of analysis.
- Detection by Fluorescence Microscopy.

Note: Cells that have bound Annexin V-FITC will show green staining in the plasma membrane. Cells that have lost membrane integrity will show red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the cell surface (plasma membrane).

Histological Changes:

Effect of diabetes on liver tissue

Control group (G1)

The result of histological examination of the liver tissue in the control group (G1) showed as a shown in figure 1.

Diabetic group 2 (G2)

The results of the histological examination of the liver tissue in the diabetic group 2 induced with alloxan showed as a shown in figure 2.

Diabetic group 3 (G3)

The results of the histological examination of the liver tissue in the diabetic group3 induced with alloxan showed as a shown in the figure 3.

The liver is composed of several lobules each lobule contains a central vein surrounded by cells named hepatocytes arranged in the form of bands and between these bands there is a bloody expanse called Sinusoids.

The results of the study showed that the diabetes mellitus induced by alloxan in males led to changes in the liver of male rats induced diabetes mellitus compared with control group and it is consistent with the studies of Al-Joubori MA (2012) that conducted on the rats

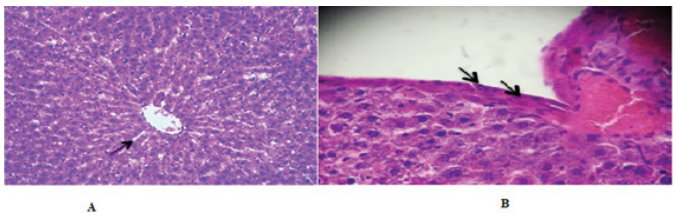

Figure 1: Histological sections of the rat liver in control group (G1) gavages with distilled water H&E (100X, 400X); (A) Histological section in the liver showing the sinusoidal capillaries among the cords (black row) H&E, 100x; (B) Histological section in the liver showing the Glisson's capsule (black row) H&E (400x).

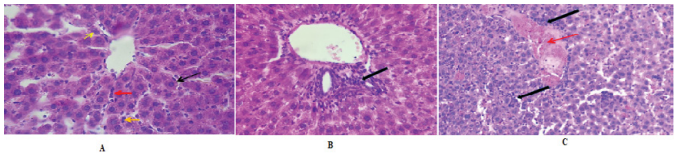

Figure 2: Histological sections of the rat liver in treated diabetic group 2 (G2) injected with alloxan 150 mg/kg for 14 (400X) H&E; (A) Histological section of the rat liver in treated diabetic group 2 (G2) showed inflammatory cells (red arrow), pyknotic nuclei (orange arrow) and binucleated cells (yellow arrow) with ballooning degeneration of hepatocyte (black arrow) H&E, 400X; (B) Histological section of the rat liver in treated diabetic group 2 (G2) showing dilatation of portal vessels with prevascular inflammatory cell infiltration (black arrow) H&E, 400X; (C) Histological section of the rat liver in treated diabetic group2 (G2) showing dilatation of central vein congestion (red arrow) with inflammatory cell aggregations (black arrow) H&E, 400x.

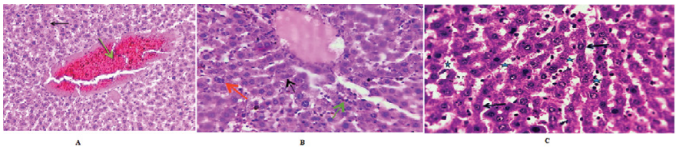

Figure 3: Histological section of the rat liver in diabetic group3 (G3) injected with alloxan 150 mg/kg for 24 days (400X) H&E. (A) Histological section of the rat liver in treated diabetic group3 (G3) showed severe congestion and dilatation of central vein (green arrow) with vacuolated hypatocytes and atrophy of sinusoid (black arrow) H&E, 400X; (B) Histological section of the rat liver in treated diabetic group3 (G3) showed marked coagulative necrosis (nuclei with karyolysis and karyorrhexis) (black arrow) with inflammatory cell proliferation (green arrow) and binucleate hepatocyte (red arrow) H&E, 400X; (C) Histological section of the rat liver in treated diabetic group3 (G3) showing hepatocelluar atrophy (arrows) and correspondingly wide sinusoids with clear pyknotic nuclei of apoptotic cells (blue stars). Hepatocytes showing different stages of mitotic divisions. The cytoplasm was granular with vesicular euchromatic nuclei with their chromatin clumped under the nuclear envelope in prophase stage of mitosis, H&E (400X).

and It showed that high blood glucose level as a result of injections with alloxan leads to a slight expansion in sinusitis and it is may be due to poor venous flow at the level of the hepatic vein or inferior vena cava as well as the emergence of several inflammatory areas and cytoplasm outbreak areas caused by damage of liver cells that occurs as a result of immunological reasons or toxic effects of alloxan induced Oxidative stress resulting from free radical aggregation causes liver cell breakdown as well as lipid peroxidation of cell membrane or mitochondrial membranes causing inflammatory Immunological response [20].

The result study was also observed presence of bloody congestion in some areas due to poor drainage due to hepatic venous obstruction, causing blood flow interrupted or disrupted to hepatic bronchial cells [21].

The onset of diabetes is accompanied by development of major biochemical and functional abnormalities in the liver, including alterations in carbohydrate, lipid, and protein metabolism, and changes in antioxidant status [22]. The prevalence of hepatobiliary diseases is increased in patients with either type 1 or type 2 diabetes [23].

Diabetes is known to be a major disorder in which oxidative stress and TNF-α production have been implicated through several lines of evidence [24]. ROS and TNF-α have been defined as an autocatalytic mechanism that can lead to programmed cell death (apoptosis). Regulation of cell death by apoptosis may be another determinant of liver structure and lesion formation [25]. It has become increasingly clear that the process of cell death by apoptosis is a relatively ubiquitous phenomenon in a variety of cell types, including hepatic cells.

In a research recently published, it is demonstrated that the diabetic state induces an increase of TNF-α and of its receptor TNF-R1 in the liver. Following TNF-α binding to the TNF-R1, an adaptor molecule (TRADD, TNF receptor associated DD protein), is recruited by the dead domain (DD) to form the first protein complex, which also includes TRAF2. This complex then dissociates from TNF-R1 and forms a different complex in the cytosol, which binds FADD (Fas associated DD protein), and then recruits caspase-8. Cleavage of pro caspase 8 allows the release of activated caspase-8 [26]. Caspase-8 can cleave Bid to form an active fragment, t-Bid. In the liver of induced diabetic rats observed an increase in the pathway that begins with the triggering of receptor TNF-R1 by TNF-α, demonstrated by increased expression and activity of caspase-8 and mitochondrial t-Bid. Pro-apoptotic protein Bid promotes initiation of the mitochondrial death pathway with release of cytochrome c, and activation of effectors caspase-3 that ultimately induce apoptosis [27]. The results clearly demonstrated that in the liver

of induced diabetic rats induces an enlargement of caspase-3 activity with the consequent increase in the apoptosis index. Besides, the anti–TNF-α treatment produced a declination in the response of receptor TNF-R1 to TNF-α showing a reduction of the cascade of events leading to apoptotic cell death [28].

An early study had demonstrated that the activation of JNK is associated with increased TNF-α induced apoptosis hepatocytes [29]. In this connection, the diabetes leads to the activation of JNK, inducing an increase of the apoptotic index.

The increase of the TNF-α level in the liver of induced diabetic rats leads to a marked up regulation of the NFκB pathway. NFκB is one of the key transcription factors involved in triggering the cascade of events that allow inflammation and different research groups have demonstrated its activation in the diabetic liver [30]. The expression of iNOS is closely related to stimulation of NFκB. Indeed, NFκB recognition sites have been identified in the promoter region of the gene encoding for iNOS.

In the liver of diabetic rats, there is increase in TNF-α due to an increased expression of iNOS, which led to a high production of NO. Similar results have been reported in different tissues by other authors [31]. It has been shown that high concentrations of glucose cause an increase in the expression of iNOS induced by cytokines in rat tissues [32]. Consistent with this, high glucose concentrations do not increase iNOS in the absence of TNF-α.

Detection of apoptosis by TUNEL assay

Apoptosis is the process of programmed cell death that occurs under normal physiological conditions, such as embryogenesis, tissue homeostasis, and immune system regulation, and can be induced by various physical and chemical stimuli. Cells undergoing apoptosis show characteristic morphological and biochemical features, which include chromatin condensation, cell and nuclear shrinkage, formation of membrane-bound cell fragments, known as apoptotic bodies, and rapid phagocytosis by neighboring cells or macrophages without associated inflammation. The biochemical hallmark of apoptosis is degradation of DNA by endonucleases, which produce double-stranded oligonucleosomal DNA fragments. These DNA fragments are 180-200 bp in size and can be separated into a ladder-like pattern on agarose gel electrophoresis. However, this method cannot provide information regarding the histological localization of DNA fragmentation at single cell level or in mixed cell populations.

TUNEL is an assay for localization of apoptotic DNA fragmentation *in situ* that was originally described in 1992. The method relies on the template-independent identification of blunt ends of double-stranded DNA breaks by TdT. The enzyme catalyzes the addition of labeled dUTPs to a 3'-hydroxyl termini of DNA ends, which can be visualized using immunohistochemical techniques. The staining kinetics of the TUNEL assay depends on reagent concentration, extent of proteolysis, and accessibility of DNA strand breaks, which vary between tissue types. Therefore, it is important to standardize the technique by using tissue sections with DNAse treatment as a positive control and without TdT treatment as a negative control of apoptosis in order to avoid false positive or negative results. Cells that have bound Annexin V-FITC will show green staining in the plasma membrane. Cells that have lost membrane integrity will show orange-red staining (PI) throughout the nucleus and a halo of green staining (FITC) on the cell surface (plasma membrane) as a show in the figures 4 and 5.

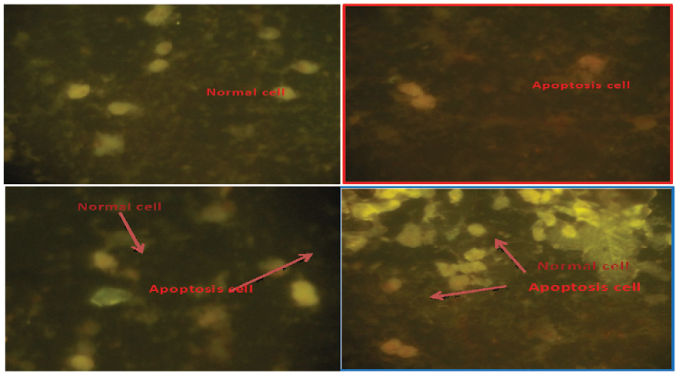

Figure 4: Tunel enzyme assay: apoptotic cell appear in green color and normal cell appear in orange - red color.

Figure 5: Necrosis cell.

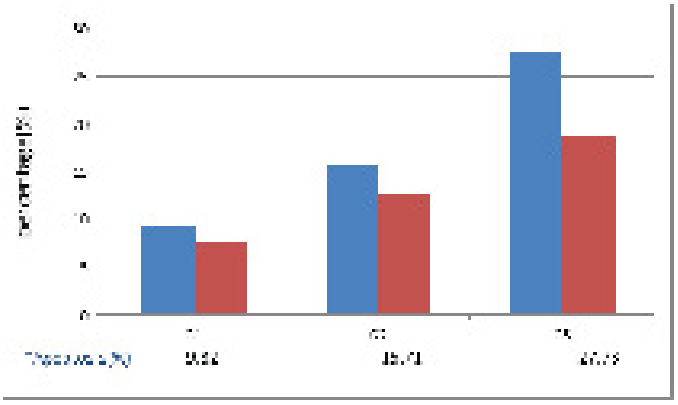

Figure 6: Apoptosis percentage according to groups: G1 (distilled water), G2 (Alloxan 150 mg/kg for 14 days), and G3 (Alloxan 150 mg/kg for 24 days).

Assessment of Apoptotic Cell Death

TUNEL assay was performed in order to characterize the effect of diabetic on induced apoptosis in the liver. The results of our study showed as in figure 6 a significant increase in apoptotic cell which is expressed by the percentage in the G2, G3 (15.71%), (27.73%) respectively compared with control group G1 (9.32%).

Diabetes is a common metabolic disorder characteristic by high elevated glucose in the blood causes hyperglycemia. The onset of diabetes is accompanied by development of major biochemical and functional abnormalities in the liver, including alterations in carbohydrate, lipid, and protein metabolism, and changes in antioxidant status [33]. Prevalence of hepatobiliary diseases is increased in patients with either type 1 or type 2 diabetes.

In the diabetes there is increase in oxidative damage and lipid peroxidation in both type 1 and type 2 diabetes as well as deficits in antioxidant defense enzymes and vitamins. It is argued that oxygen, antioxidant defenses, and cellular redox status should be regarded as central players in diabetes [35].

On the other hand, it was established that hyperglycemia increases mitochondrial reactive oxygen species (ROS) production, which could represent a key event in the development of diabetes complications [35]. The initial cellular response to high glucose challenge is the generation of ROS, which rapidly induces apoptotic cell death. It is known that high D-glucose induces endothelial apoptosis through activation of the Bax caspase proteases pathway. The effectors of apoptosis are now well known to be represented by a family of intracellular cysteine proteases known as caspases.

A feature of apoptosis that impinges on caspases is altered mitochondrial function characterized by a reduction in the electrochemical gradient across the mitochondrial membrane and release of mitochondrial cytochrome c to cytoplasm and it is inhibited by the presence of Bcl-xL in these organelles [36].

Translocation of pro apoptotic BAX protein into the mitochondrial membrane is accompanied by a significant increase in caspase-3 (CASP3) and caspase-9 (CASP9) activities and then caused finally cell death (apoptosis) [37-39].

Conclusions

From the results of this study we conclude that the elevation in the serum glucose level could be attributed to insulin deficiency caused by selective destruction of β-cells of islets of Langerhans by alloxan which in turn causes additional changes in the liver of infected animals due to high production of Oxidative stress resulting from free radical aggregation causing liver cell breakdown as well as lipid peroxidation of cell membrane or mitochondrial membranes and this leads to poor venous flow at the level of the hepatic vein or inferior vena cava and expansion in sinusitis and formation of inflammatory areas. The induced diabetes led to increase in apoptotic cell death of the liver due to generation of mitochondrial reactive oxygen species (ROS) which pay represent a key event in the development of diabetes complications resulting the initial response to liver apoptosis.

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