Examination of Immuno-pathological changes in Diabetic Mice

Sabrin Ibraheem Mohsen1, Taghreed Jabbar Humadi1, Muna Sachit Hashim1*

1. College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

* Corresponding author:
Dr. Muna Sachit Hashim
College of Veterinary Medicine,
University of Baghdad,
Baghdad, Iraq.
E-mail: sachitmuna@yahoo.com

Background: Diabetes mellitus is a metabolic disorder a major health concern today whose prevalence has continuously increased of the global population. Alloxan-induced diabetes is used model to induce Type I diabetes mellitus in the experimental animals are one of the best strategies for the relative with immune responses of diabetic mice, It has been found toxic to pancreatic beta cells due to structural similarity to glucose analogues that is capable of inducing type I diabetes mellitus in experimental animals. Objectives: Examination immune-pathological changes in different organs after inducer diabetic in mice and determination of cellular immunity through 2 tissue markers (TNF, Glucagon).

Methods: study design was animals randomized, in order to study the patho-immunological effects of induction of diabetes in mice (tissue damage biomarkers). Twenty one mice(100-120 g) were used in this experiment, those subdivided into three groups, first group (diabetic group) contained seven mice were injected intraperitonial at daily doses for 6 weeks of alloxan (Alloxan monohydrate 150mg/Kg at single dose of 0.1ml/10mg B.W.), second group (immunized group) contained seven mice were stimulated immune response by vaccine of Brucella melitens is Rev.1 at dose 0.2ml/mouse subcutaneously at 2 doses between 2 weeks interval, third group (control group) contained seven mice were injected at daily dose of physiological saline solution. Results: Immunological analysis were done by use two cellular marker (TNFα alpha and Glucagon). Diabetic mice appear grossly very sick after Alloxan's injection and microscopically necrotic tissues with vacuoles within cytoplasm due to degeneration, mainly in liver; kidney as well as spleen showed lymphoid atrophy and skin showed loss of hair follicles with thickening epidermis and dermis layers. In immunized group mice appear similar to control group in activity grossly, and microscopically low level of pathologic changes were appeared. Conclusion: Microscopic immune-pathological investigation of liver; kidney as well as spleen tissues showed marked significant decrease appear degenerated with a significant increase level of cytokine (TNF-α) and decrease of glucagon which could have the ability to regenerate tissue, cytokine repairing of necrotic tissue in early duration of diabetes metabolic. Pathological and immunological methods of inducing type II diabetes mellitus in experimental animals by drug-induced diabetic (alloxan) in skin, spleen, kidney and liver, that alloxan caused of immunopathological changes from skin, spleen, kidney and liver that lead to associated of someone of cytokines activation in the body with immunization.

Keywords: Alloxan, Diabetes mellitus type 2; TNF-α.

Introduction

Diabetes mellitus was the most common endocrine disease accompanied with metabolic disorders [1,2]. This disease leads to different pathological and physiological effects in different organs [3]. One of the most methods to induce experimental diabetes mellitus was chemical induction by Alloxan [4]. DM is a metabolic disorder that is characterized by chronic high blood glucose level that leads to complications in the eyes, kidneys, heart, vessels and nerves [5]. This chronic complex disease requiring permanent medical care with multifactorial risk reduction strategies beyond glycemic control [6].

It is a urea derivative which may cause necrosis of the β-cells of pancreatic islets. It has been used to produce experimental diabetes in animals such as mice, rats, rabbits, and dogs with different dose of alloxan used [7,8]. Inhibition of glucokinase enzyme, generation of free radicals and disturbances in intracellular calcium homeostasis [9,10]. When administered parenterally, i.e., intravenously, intraperitoneally or subcutaneously, the dose of alloxan is required for inducing diabetes depends on the animal species, route of administration and nutritional status [11, 12].

TNF-α is implicated in the destruction of B cells from in experimental animals studies on isolated islets and has profound inflammatory effects in auto reactive T lymphocytes [15, 16]. TNF-α inhibits insulin transduction, and has an effect on glucose metabolism [17]. Disturbances in the TNF-α metabolism have been implicated in metabolic disorders, such as obesity and insulin resistance, indicating that perturbations of TNF-α metabolism may affect the onset of type 2 diabetes mellitus and the progression of the disease [19,20].

Glucagon is a hormone that is produced by alpha cells known as the islets of Langerhans in a part of the pancreas. Glucagon plays an important active role in allowing the body to regulate the utilization of glucose and fats. Glucagon is released in response to low blood glucose levels and to events whereby the body needs additional glucose, such as in response to vigorous exercise [26, 27]. Glucagon serves to keep blood glucose levels high enough for the body to function well. When blood glucose levels are low, glucagon is released and signals the liver to release glucose into the blood. In people with diabetes, glucagon’s presence can raise blood glucose levels too high. The reason for this is either because not enough insulin is present or, as is the case in type 2 diabetes, the body is less able to respond to insulin [28].

The aim of presented study for examination immune-pathological changes in different organs after inducer diabetic in mice and determination of cellular immunity.

Materials and Methods

Experimental animals

An experiment was conducted in 30-mixed white mice of uniform age (4 weeks) and about (18-24) gram weighting were used in the present study. All animals were housed in polyethylene (50cm×25cm×25 cm) home cages, with saw-dust covered floors at the Animal House of, Faculty of veterinary medicine, Baghdad University.

The animals were kept at the room temperature with a natural lighting cycle (12-h dark/light cycle). They were fed standard laboratory and given fresh tap water through a glass bottle with a capillary dropper fixed to the wall of a cage in a position to be available for mice, the cages were cleaned every day. Procedure of the experimental animals was in accordance with ethical standards and guidelines for toxicological research.
All the present experiments were reviewed and approved by Animal Ethics Committee.

**Experimental groups and design of work:**

These mice were divided into three groups of animals for each group composed of 10 mice:

**Group 1:** allooxan induced diabetic mice, mice were injected with allooxan solution (0.1ml/10mg) / intarperitoneally (Rashid et al., 2013).

**Group 2:** Immunazing mice with attenuated *Brucella melitensis* Rev.1 (0.2ml/mouse)S/C) was injected with allooxan solution (0.1ml/10mg) / intarperitoneally.

**Group 3:** Control group, was injected with isotonic saline solution (0.9% NaCl)/ intraperitoneally (0.1ml/10mg body weight).

**Table (1):** Determination's blood glucose level, of each group of experimental animals

<table>
<thead>
<tr>
<th>Animals group</th>
<th>Treatments</th>
<th>Glucose level mg/dL (rang)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: diabetic mice No=10</td>
<td>Alloxane (0.1ml/10mg BW) IP, Single dose</td>
<td>200-400 *</td>
</tr>
<tr>
<td>Group 2: Immunized group No=10</td>
<td>Vaccine of <em>Brucella melitensis</em> Rev.1, at dose of (0.2ml/mouse)subcutaneously + Alloxane (0.1ml/10mg BW)</td>
<td>120-250 **</td>
</tr>
<tr>
<td>Group 3: Control No=10</td>
<td>Isotonic solution Ip</td>
<td>75-100</td>
</tr>
</tbody>
</table>

Significantly different from corresponding control as assessed by analysis of variance: *p< 0.05; ** p <0.01

Mice groups were sacrificed after 3 weeks of induction by allooxan, liver, kidney, spleen and skin tissue were collected for histopathology immune assays to detection of deposition of TNF-α in tissues by using (TNF-α mice kit, USA Co.), which has an inter-assay cut off. Alloxan monohydrate (0.1ml/10mg) has been prepared in 10% saline solution and injected intraperitoneal at single dose of (0.1ml/10 mg B.W) weekly for three weeks, blood glucose level of these mice was estimated 72 hr. after alloxan administration, and diabetes was confirmed by blood samples collected from the tip of the tail using a blood glucometer (Accu Sure, 4 Taiwan). Animals with blood glucose level equal to or more than 200 mg/dL were declared diabetic [1,16] Tissue samples from different organs were fixed with formalin (10%) and prepared slides after processing according to [20].

**Antigen (Vaccine)**
The antigen used in the present study is a vaccine of freeze-dried suspension of live attenuated *Brucella melitensis* Rev.1, Elberg strain (3-8) month's age. At concentration of (1-2×10^6 C.F.U). Origen of Vetal Turkey. Company, The Ag was used in a dose of (0.2ml/ mouse) according to (21). The antigen was given in 2 doses. The first at the beginning of the experiment i.e. before VCR injection, the 2^nd^ dose was given 2 weeks before scarifying the animals in each different intervals to each subgroup.

**Induction of diabetes in mice**

Mice were subjected to fasting for twenty four hours free access to water. Alloxan monohydrate (Sigma-Aldrich Chemical Co., USA) alloxan powder was prepared by dissolving it in normal saline 0.9% NaCl injected intraperitontial at a dose (120 mg/kg body weight) into the fasted mice (Rashid et al., 2013) storage temp.(2-8°C) in refrigerator. Blood glucose level was determined after 48 hour of injection, using blood sugar measurement (Accu Check china). Mice with blood glucose level 200 mg/dl were supposed to be diabetic (Bahar, 2011), these mice were separated at specific cadges.

**Histopathological examination**

After collection of tissue for immune assays, mice were scarified and abdominally dissected. The skin, spleen, kidney and liver were removed from each mouse and then washed within a normal saline solution (0.9% NaCl) for the removal of the blood, which might affect the process of fixation. Samples should be fixative (10% formalin pH=7) for 24 hours after dissected. The tissues were embedded in paraffin wax after they were drying and clearing, and dyed with hematoxylin and eosin (H&E) dye(Kiernan, 2012). The dyed sections were examined by using electron microscope and photographed by digital microscope camera. All images of skin, spleen, kidney and liver tissues were using objectives 40x and 10x for optical system.

**Statistical analysis**

All the grouped data were statistically evaluated and the significance of changes caused by various treatments was determined using (Essential Statistics Terms, 2017). The statistical significance was set at P <0.05 and 0.01. Histopathological analyses were performed by a pathologist who should be blinded to groups allocation.

**Results and Discussion**

**Histopathological examination:**

**First group (inducer Diabetic group):**

1-Skin: diabetic mice showed skin loss hair in local area so we take samples for histopathological examination and we found atrophied hair follicles at microscopically examination as appear in figure 1.
Figure 1: Skin of mouse injected with Alloxan's intraperitoneal at dose (0.1ml/10 mg B.W) appears A: Skin collagen fibers thickening within dermis and epidermis, B: loss of hair follicles and atrophied sebaceous gland. C: proliferation of collagen and fibrous elements. H & E.10X.

2-Kidney: diabetic mice showed severe degenerated kidney which appear very swollen due to hydropic degeneration with loss of epithelial lining renal tubules with dark nuclei as a first step of necrosis figure 2.

Figure 2: Kidney of mouse injected with Alloxan's intraperitoneal at dose (0.1ml/10 mg B.W) appears A: renal tubules swelling and attached to each other's and others dilated with huge lumen (arrow) . B: narrowing luminal space with protruded epithelial lining. H & E.10X. C: closed lumen and star shape formation and other dilated. H & E.40X.

3- Liver: diabetic mice showed severe degenerated kidney which appear very swollen due to hydropic degeneration with loss of sinusoidal space with dark nuclei as a first step of necrosis figure 3.

Figure 3: Liver of mouse injected with Alloxan's intraperitoneal at dose (0.1ml/10 mg B.W) appears A: dilated central vein within atrophied hepatic lobe and cloudy swelling within hepatocytes. H&E.10X. B: fragmentation of hepatocytes nuclei within each lobules of hepatic parenchyma refer to necrosis &E.40X.

4-Spleen: Spleen of diabetic mouse appear red in colour due to hemorrhagic changes associated with yellowish discoloration as results of hemosiderin deposition from destructive RBC, capsule widening and white pulp depletion also appear. Figure 4
First group (inducer diabetic group):

After application of immunological procedures specialized for (TNFα alpha and Glucagon) tissue's marker on different organs (skin, liver, kidney and spleen). Results reported by using lighted microscopic under 1000x power lenses. Skin as well as liver, kidney and spleen looks dark brown after interaction of inflammatory cellular components with TNFα extracellular at cell membranes. As showed in Figure 5, 6, 7 and 8. Alloxan is widely used to induce diabetes in experimental animals that causes damage of pancreatic β-cells. It has been reported to massive increase in cytosolic calcium concentration cause rapid destruction of pancreatic β-cells and thus hyperglycemia is occurred (Rohilla & Ali, 2012). Inhibition of glucokinase enzyme is involved in the toxic action of alloxan on pancreatic β-cells (Dhanesha et al., 2013). Alloxan was also shown to induce diabetes agreeing with Ebel D., et al (2003). Galagudza et al. have found that diabetic mice for four weeks were more damage for skin, liver, spleen and kidney compared to control mice. However, diabetes mellitus induced by alloxan interferes with the protective effect of other species as some as Babiker F. A, et al.(2012).

Figure 5: A: Skin interaction against TNFα alpha marker appears dark brown, B: skin interaction against Glucagon marker appear dark blue. These dark discolorations refer to immune response. Dap. 400X

Figure 6: A: Liver TNFα alpha deposition dark brown: deposition of Glucagon marker as dark blue over hepatocytes. Dap.1000X.
Tissues pieces of skin; kidney; liver and spleen of vaccinated mice with vaccine of freeze-dried suspension of live attenuated *Brucella melitensis* Rev.1, Elberg strain (3-8) month's age. In a concentration of (1-2×10⁶ C.F.U). Origen of Vetal, Turkey. Company, at dose of (0.2ml/ mouse) were examined under light microscope after well processing in histopathological laboratory. Skin sections appeared low thick due to low hyper keratinization in epidermis and dermis layer; kidney appear with thick intima layer of congested blood vessels, liver look slightly degenerated of in lobes has dilated blood vessels, spleen looks with amyloids deposition as a results of immune response and white pub appear dark in colour as result of hyperplasia of lymphocytes, in general histopathological finding of immunized group we found low pathological lesions in examined organs.
Figure 9: A- skin looks with low keratinization in epidermis underlying tissue appear with collagens fibers slightly thick (arrow).H&E400X. B: Kidney showed thick congested blood vessels with normal arrangements of cortex. H&E 100X. C: Liver looks with normal structures unless dilated blood vessels (arrow). H&E 100X. D: Spleen looks with amyloidosis(arrow) and widening white pub. H&E 100X.

Immunized group: After application of immunohistochemistry procedures of two different immune markers, TNFα alpha and Glucagon tissues marker according to producer company orders. Results were calculation depend on antigen antibody reaction over cell membrane which appear as small dark dot. Tissues of spleen; Kidney; liver and skin appear loading this reaction in immunized group with low demarcation as comparison to diabetic group. As shows in (Figure 10.11,12 and 13).

The pathological change observations in the present work confirmed the control group compared with inducer diabetic group. The pancreatic tissue of inducer diabetic mice demonstrated damage in the Langerhan’s islet cells, decreasing islets size, decreasing in β-cell number. These data was in agreement with El-Esawy et al. (2016). The blood sugar level was increased daily with TNF-a blood level was increased, the degeneration in the islets showed in the histopathological results was significantly increased in diabetic inducer mice, histopathological data obtained from the current study were also in consistent with Hadi et al. (2016). Vaccination was a determinant for brucellosis control and eradication programs. Efforts to find out the principal characteristics of the immune to establish an ideal vaccine. The definition of immune markers correlated with protection, by mathematical modeling or evaluation of the immune response agreement with Elaine MS Dorneles et al. (2015).

Figure 10: Spleen of immunized mice with live attenuated Brucella melitensis Rev. 1 vaccine (0.2ml/mouse) S/C. A: Glucogan deposition as dark blue. B: TNF marker above lymphocytes membrane as dark brown color. Dap.1000X.
Hashim et al. (2019): Immuno-pathological of diabetes in mice December 2019 Vol. 22(10)

Figure 11: Skin of immunized mice with live attenuated Brucella melitensis Rev. 1 vaccine (0.2 ml/ mouse) S/C. A: Glucogen deposition as dark blue within dermis and epidermis. Dap. 400X. B: TNF deposit very thick in epidermis and low thickness in dermis. Dap. 1000X.

Figure 12: Liver of immunized mice with live attenuated Brucella melitensis Rev. 1 vaccine (0.2 ml/ mouse) S/C. A: Glucogen appear dark blue intracellular near hepatocyt's nuclei. Dap 1000X. B: TNF make replacing of intracellular and extracellular space as dark brown discoloration. Dap 1000X.

Figure 13: Kidney of immunized mice with live attenuated Brucella melitensis Rev. 1 vaccine (0.2 ml/ mouse) S/C. A: Glucogen deposition as dark blue. Dap 400X. B: renal tubules appear complete replacing with TNF marker as brown color. Dap. 1000X.

Control group: Animals from control group also submitted to the same examination procedures: Histopathological Examination: Tissues samples of liver and kidney and spleen and skin appear in normal structures and normal cellular and nuclear staining as shows in Figure 14.
Figure 14: Organs of control group treated with isotonic solution IP: 

A: Liver appear in normal arrangement of hepatocytes within lobules acentric with central vein. 400X H&E. 

B: Kidney appear normal arrangements of nephron. 400X H&E. 

C: Spleen white pulp and red pulp appear normal. 100X H&E. 

D: Skin appear with normal hair follicles (arrow). 100X H&E.

Control group: Tissues from control group appear with no or weak immune reaction as appear in Figure 15.

Figure 15: Immunohistochemistry procedures on tissues spacemen’s of control group shows 

A: Spleen 
B: Kidney 
C: Liver 
D: Skin. 

There no interaction in cellular componants. Dap. 400X.
Conclusion

Microscopic immune-pathological investigation of liver; kidney as well as spleen tissues showed marked significant decrease appear degenerated with a significant increase level of cytokines (TNF-α) **and decrease of glucagon which could** have the ability to regenerate tissue, cytokines repairing of necrotic tissue in early duration of diabetes metabolic. Pathological and immunological methods of inducing type II diabetes mellitus in experimental animals by drug-induced diabetic (alloxan) in skin, spleen, kidney and liver, that alloxan caused of immunopathological changes from skin, spleen, kidney and liver that lead to associated of someone of cytokines activation in the body with immunization.

Conflicts of interest: None of the authors have any conflicts of interest relevant to this research subject.

Ethical Approval

Ethical Committee at the University of Baghdad, college of College of Veterinary Medicine, Iraq approved the study.

references


