



## The Potential Protective Effects of Tetrahydrobiopterin on Cadmium-Induced Pancreatic Changes in Male Rats

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### Abstract

Cadmium (Cd) is a widespread environmental and industrial pollutant. It accumulates in the pancreas and could influence its endocrine and exocrine functions. Tetrahydrobiopterin (BH4) is essential for various processes, and present in all tissues of higher organisms. This study was designed to investigate the effect of BH4 on the acute pancreatic damage induced by Cd and detect its mechanism(s) of action. Thirty rats were randomly divided into three groups (10 rats each). Control: received saline, Cd: received (single dose of CdCl<sub>2</sub> 4 mg/kg, i.p.) and BH4+Cd: received (single dose of BH4 20 mg/kg, i.p.) one hour before single dose of CdCl<sub>2</sub> (4 mg/kg, i.p.). The  $\alpha$ -amylase, lipase, glucose, insulin and interleukin 6 (IL-6) levels were measured in serum and intercellular adhesion molecule-1 (ICAM-1), malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured in pancreatic homogenate. Histopathological examination of pancreas was done. BH4 improved pancreatic functions, where  $\alpha$ -amylase, lipase, glucose and IL-6 levels were significantly decreased while insulin levels were significantly increased in serum. Pancreatic damage was ameliorated as evident by significant decrease of ICAM-1 and MDA and significant increase of SOD levels in pancreatic homogenate. Also, the disturbed pancreatic tissues were ameliorated. In conclusion, BH4 induced improvements in pancreatic tissue and functions in cadmium-exposed rats. Part of BH4 beneficial effects could be attributed to anti-oxidative and anti-inflammatory activity.

### Keywords

- Tetrahydrobiopterin,
- Cadmium,
- Inflammation,
- Oxidative Stress

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## INTRODUCTION

Cadmium (Cd) is one of the widely used heavy metals and implicated in many industrial applications like electric batteries, electronic components, pigment, and fertilizer and considered as environmental pollutant [1]. Major sources of cadmium exposure include the diet, in particular rice, cereals, potatoes, and other root vegetables, and also smoking as cadmium in tobacco smoke is effectively absorbed in the lungs [2]. It accumulates in various organs, the kidneys, liver, testes, pancreas, thyroid, salivary glands, bone and central nervous system [3].

Cadmium could influence both endocrine and exocrine functions of pancreas [4]. Also, it leads to necrosis, degeneration and degranulation of beta cells causing an increase in serum glucose level [5]. The initial acinar cell damage in the early stage of acute pancreatitis of any etiology is caused by a hypersecretion of pancreatic proteolytic enzymes [6]. Cadmium induces tissue injury through creating oxidative stress and decreasing the biological activities of some antioxidant enzymes [7]. In addition, it can cause release of inflammatory mediators and enhance expression of adhesion molecules that initiate a cascade of cellular and humoral responses leading to inflammation [8].

Tetrahydrobiopterin (BH4) is present in probably every cell or tissue of higher organisms and plays a key role in a number of biological processes and pathological states associated with monoamine neurotransmitter formation, cardiovascular and endothelial dysfunction, the immune response and pain sensitivity [9]. BH4 is biosynthesized from

guanosine triphosphate (GTP) [10]. BH4 is an essential cofactor for all three nitric oxide synthase (NOS) isoforms (endothelial, neuronal, and inducible), aromatic amino acid hydroxylases, and alkylglycerol monooxygenase [11, 12]. Also, It is involved in the biosynthesis of neurotransmitters, including epinephrine, norepinephrine, dopamine, and serotonin [13].

Tetrahydrobiopterin improved the NO-mediated endothelial function in patients with vascular disease states, such as hypercholesterolaemia [14], Type 2 diabetes [15] and overt coronary atherosclerosis [16]. It is essential in prevention of lethal murine pancreas ischemia reperfusion injury [17]. BH4 reduces tissue injury following ischemia-reperfusion injury after kidney, liver, lung and heart transplantation [18]. In addition, BH4 can reverse inflammation-induced impairment of the endothelium [19]. However, in pathological states in which BH4 bioavailability is reduced (e.g., oxidized by increased levels of free radicals, such as superoxide and peroxynitrite), NOS becomes dysfunctional and its activity “uncoupled” to favor superoxide production. This imbalance in NO/superoxide production results in oxidative stress, a major contributing factor in a variety of vascular dysfunction associated with hypertension, ischaemic reperfusion injury and diabetes [9]. The interest in the role of BH4 continues to grow. Therefore, the present study was designed to study the effect(s) of BH4 on the acute pancreatic damage induced by cadmium and demonstrate the possible mechanism(s) of its action.

## MATERIALS AND METHODS

### *Experimental animals*

A total number of thirty adult (about twelve-weeks old) male albino rats weighing 140–180 g were obtained and maintained in Animal house of Faculty of Medicine, Assiut University. They were kept in well ventilated room at temperature of (23±3°C) under natural light/dark cycle and were allowed free access to standard rat chow and water. The experimental procedures were carried out according to Guidelines of Care and Use of Laboratory Animals and approved by Ethical Committee at Faculty of Medicine, Assiut University, Egypt.

### Chemicals

(6R)-5, 6, 7, 8-Tetrahydrobiopterin dihydrochloride (BH<sub>4</sub>) and cadmium chloride (CdCl<sub>2</sub>) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA.

### *Experimental Design*

Rats were randomly divided into three equal experimental groups (10 rats each). Control group: were given 1 ml of normal saline (0.9% NaCl) injected intra-peritoneal (i.p.). Cadmium-treated group (Cd): received single dose of 4 mg/kg body weight of CdCl<sub>2</sub> [20] which was dissolved in normal saline and injected i.p. into the rats. BH<sub>4</sub>+Cd-treated group (BH<sub>4</sub>+Cd): received single dose of BH<sub>4</sub> (20 mg/kg, i.p.) [21] and subsequently exposed to single dose of CdCl<sub>2</sub> (4 mg/kg, i.p.) one hour after the BH<sub>4</sub> treatment.

### *Collection of samples and biochemical Analysis*

After 24 h from Cd exposure, 2 ml blood were collected in glass tubes from orbital sinus and whole blood was centrifuged after clotting, and the serum was separated and the samples were

maintained at -20 °C until used. The animals were sacrificed, then the pancreas was obtained from each animal, part was stored at - 80°C for subsequent biochemical analysis and the other part fixed with 10% formalin phosphate and processed for haematoxylin and eosin (H&E) staining for histological examination.

### *a- Estimation of biochemical parameters in the serum*

Serum  $\alpha$ -amylase and lipase were measured by colorimetric enzyme assay kits (Lab-Care Diagnostics, INDIA). Serum glucose level was determined using the colorimetric analysis (Abcam, Cambridge, MA, United States). Ultra-sensitive rat-specific ELISA kit (Crystal Chem, USA) was used for insulin assay. Serum IL-6 was measured by (BioSource International, Camarillo, California, USA).

### *b- Biochemical parameters in pancreatic tissue*

Part of the frozen pancreatic tissues was homogenized in 50 mM phosphate buffer (pH 7.4) by means of a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelheim, Germany) on an ice cube. The homogenates were centrifuged at 7530g in 4 °C for 10 min. The supernatant of tissue homogenate was used for determination of: 1) The presence of MDA, a biomarker of lipid peroxidation by the method described by Al-Fawaeir et al. [22]. 2) SOD activity as previously described by Aydin et al. [23]. The results were expressed in relation to the protein content. 3) The other part of the frozen pancreatic tissues was taken and homogenized with ICAM-1 reaction buffer supplied with the kit. The supernatants obtained after centrifugation were used to determine ICAM-1 using a rat ELISA Kit (Bosde

Biotechnology, Wuhan, China). Protein content of the supernatants was determined using Lowry et al. [24] method.

### *Histopathological examination*

Pancreatic tissues were fixed with 10% neutral formalin phosphate buffer, dehydrated through a graded alcohol series and embedded in paraffin, then were cut into 5-7  $\mu\text{m}$  sections and stained with haematoxylin and eosin according to Drury and Wallington [25]. The sections were examined under light microscopy.

### *Statistical analysis*

Statistical analysis was performed using the GraphPad Prism software version 3 (GraphPad Software, San Diego California, USA). The results were presented in the form of mean  $\pm$  standard deviation (SD) for ten rats in each experimental group. One way analysis of variance (ANOVA) with Bonferroni Multiple Comparison test was

done to compare between the studied groups. P-values  $< 0.05$  were considered as significant.

## **RESULTS**

### *1-Biochemical markers in serum*

The levels of serum amylase and lipase enzymes were significantly increased in cadmium treated compared to the control group ( $P < 0.001$  for each). However, in BH4+Cd group there are significant lower levels of amylase and lipase versus Cd group (for amylase  $P < 0.01$  and for lipase  $P < 0.05$ ). Comparing to control amylase and lipase enzymes were still significantly higher ( $P < 0.05$  for each) in BH4+Cd group (Table 1).

Serum level of glucose was significantly increased in Cd group as compared to the control group ( $P < 0.001$ ). Treatment with BH4 in BH4+Cd group lead to significant lower level of glucose as compared to Cd group ( $P < 0.01$ ). There was non-significant change in glucose level in BH4+Cd group when compared with control (Table 1).

**Table 1: Serum levels of  $\alpha$ -amylase, lipase, glucose and insulin in the studied groups.**

	<b>Control n = 10</b>	<b>Cd n = 10</b>	<b>BH4+ Cd n = 10</b>
<b><math>\alpha</math>-amylase (U/L)</b>	62.60 $\pm$ 5.34	76.2 $\pm$ 3.19 ***	68.6 $\pm$ 4.25 *, ##
<b>Lipase (U/L)</b>	24.40 $\pm$ 3.03	29.8 $\pm$ 1.54 ***	27.0 $\pm$ 1.60 *, #
<b>Glucose (mmol/L)</b>	9.10 $\pm$ 1.01	11.6 $\pm$ 1.20 ***	9.9 $\pm$ 1.20 ns, ##
<b>insulin (nmol/L)</b>	0.41 $\pm$ 0.04	0.31 $\pm$ 0.04 ***	0.37 $\pm$ 0.03 *, ##

Data are the mean  $\pm$  SD. \*  $P < 0.05$ , \*\*\*  $P < 0.001$ , ns: non significant as compared to control group.

#  $P < 0.05$  and ##  $P < 0.01$  as compared to Cd treated group. Cd: Cadmium chlorid, BH4: Tetrahydrobiopterin.

Regarding serum insulin level in different groups, cadmium-exposed group shows a significant reduction of insulin level versus control group ( $P < 0.001$ ). Of interest, BH4 treatment in BH4+Cd group caused a significant increase of the insulin level versus cadmium exposed group ( $P < 0.01$ ), but

still there was a significant decrease of insulin hormone in comparing to control group ( $P < 0.05$ ) (Table 1). IL6 level of the Cd group was significantly increased when compared to the control ( $P < 0.001$ ). Co-administration of BH4 and cadmium in BH4+Cd group resulted in significant

reduction of IL6 compared to Cd group ( $P < 0.01$ ). IL6 level of BH4+Cd group was significantly increased as compared to control ( $P < 0.05$ ) (Fig 2 b).

**2-Biochemical markers in pancreatic tissue homogenate**

According to (Fig 1 a, b) which demonstrated that in pancreatic tissue homogenate MDA was significantly increased ( $P < 0.01$ ) and SOD was

significantly decreased ( $P < 0.01$ ) in cadmium treated group compared to the control group. Treatment with BH4 in group III resulted in a significant lower level of MDA ( $P < 0.05$ ) and a significant higher level of SOD ( $P < 0.05$ ) as compared to Cd group. There were normalization of both MDA and SOD in pancreatic tissue homogenate as compared to control.

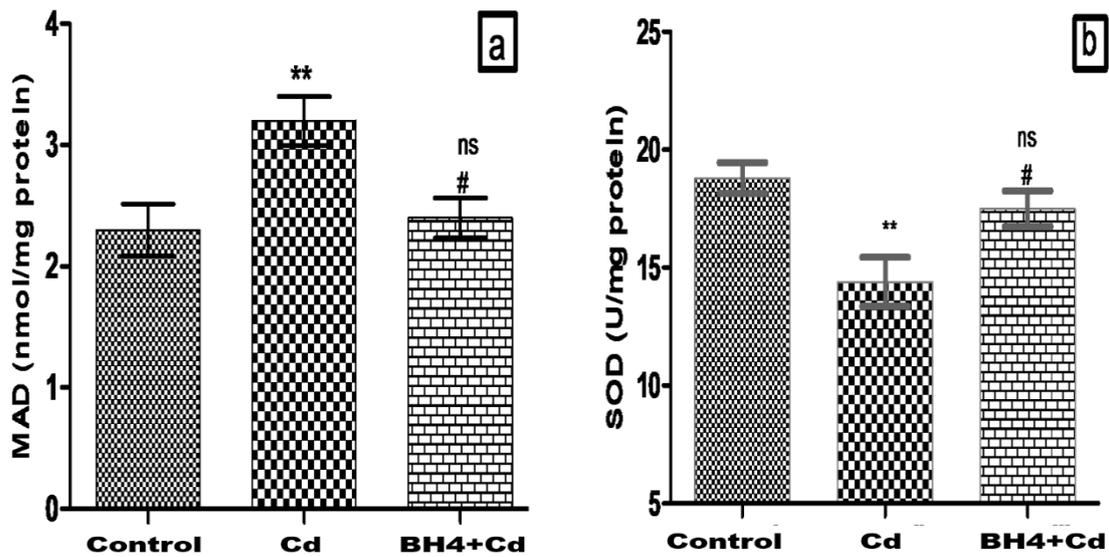


Fig.1. Levels of a: MDA (nmol/mg protein) and b: SOD (U/mg protein) in pancreatic tissue homogenate of the studied groups. \*\*  $P < 0.01$ , ns: non significant, as compared to group I. # $P < 0.05$  as compared to group II. MDA: malondialdehyde. SOD: superoxide dismutase

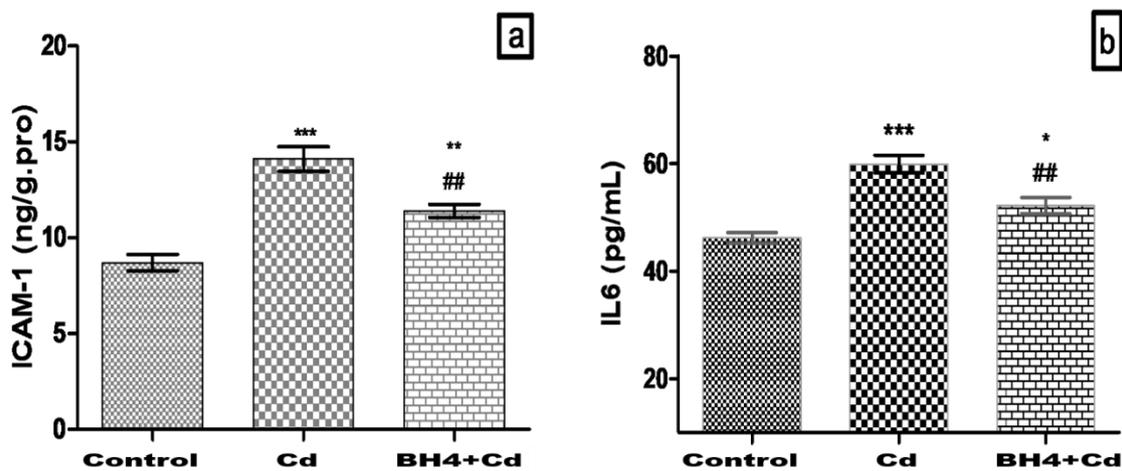


Fig. 2. Levels of a: ICAM-1(ng/g.pro) in pancreatic tissue homogenate and b: IL6 (pg/mL) in serum of the studied groups. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , as compared to group I. ## $P < 0.01$  as compared to group II. ICAM-1: intercellular adhesion molecular-1. IL 6: interleukin 6

The level of ICAM-1 in pancreatic tissue homogenate was significantly increased in cadmium treated (Cd group) compared to the control group ( $P < 0.001$ ). However, BH4+Cd group had a significant lower level of ICAM-1 in pancreatic tissue versus Cd treated group ( $P < 0.01$ ). Comparing to control ICAM-1 in pancreatic tissue homogenate was still significantly higher ( $P < 0.01$ ) in BH4+Cd group (Fig 2 a).

### **3-Histopathological results**

#### **Control group:**

The pancreas of control animal shows islets of Langerhans surrounded by many serous acini with very small lumens. The interlobular ducts lined by columnar epithelia. Cells of the islets of Langerhans are clumped masses of polygonal or rounded, smaller and more lightly stained than the surrounding acinar cells, arranged in cords separated by capillaries. The acinar cells of the acini are columnar containing basal nuclei. The supranuclear and apical cytoplasmic spaces are packed with secretory granules. Their nuclei are large and lightly stained. The basal regions of the gland cells are stained blue-violet (Fig 3 a).

#### **Cd group:**

The pancreas of Cd treated animal shows disturbed acinar pattern with narrow acinar lumen indicating little amount of secretion. Cellular infiltrations between the acini are clearly obvious. The islets of Langerhans cells show pale stained nuclei (lighter than control). Dilated capillaries between the islets cells are clearly obvious (Fig 3 b).

#### **BH4+Cd group:**

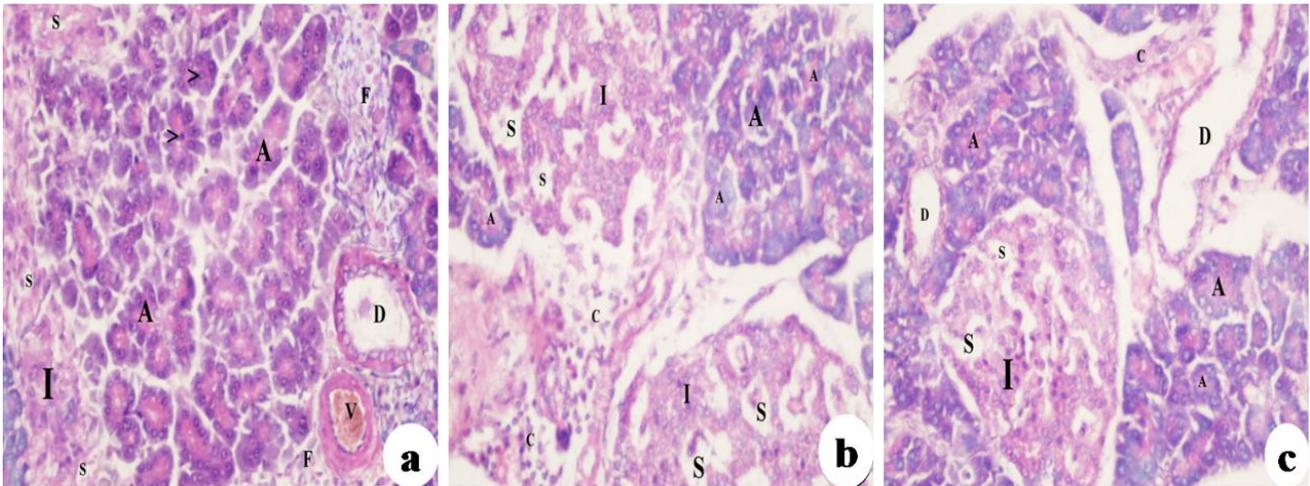
The pancreas of BH4 and Cd treated group shows acini more or less similar to the control. Cellular infiltrations are little. The islets of Langerhans

cells showed pale stained nuclei (lighter than control). Dilated capillaries between the islets cells are clearly obvious but less than that of Cd treated alone (Fig 3 c).

### **DISCUSSION**

The present study showed significant increase in the serum levels of  $\alpha$ -amylase, lipase and glucose and significant decrease in serum insulin level following cadmium administration compared to the control group indicating pancreatic damage. These findings is consistent with Khorasgani et al. [26] who found that cadmium had exerted a toxic effect on pancreatic tissue which lead to extrusion of pancreatic lipase and amylase into the plasma. Lei et al. [27] found that Cd affects carbohydrate metabolism by injuring the Langerhans islet beta cells and reducing insulin secretion leads to hyperglycemia.

The present data revealed that BH4 administration decreased significantly the levels of  $\alpha$ -amylase and lipase and glucose and increased significantly insulin level. These findings support those of Sugiyama et al. [28] who showed that the increases in the serum amylase level were significantly attenuated by the administration of BH4. Abudukadier et al. [21] demonstrated that BH4 has a glucose-lowering effect by suppressing hepatic gluconeogenesis in an endothelial nitric oxide synthase dependent manner and ameliorates glucose intolerance as well as insulin resistance in diabetic mice



**Fig. 3.** Representative photograph of rat's pancreas of: (a) Control group showed islets of Langerhans (I) surrounded by many serous acini (A). The centroacinar cells (arrow head) inserted into the acinar lumen. The interlobular ducts (D) lined by columnar epithelia. Cells of the islets of Langerhans arranged in cords separated by fenestrated capillaries (S). The acini are surrounded by connective tissue with fibroblasts (F). The ducts and blood vessels (V) are located in connective tissue. (b) Cd group showed that the acini are disturbed in shape with narrower acinar lumen. Cellular infiltrations (C) and dilated capillaries between the islets (S) cells are clearly obvious. (c) BH<sub>4</sub>+Cd group showed that the acini are more or less similar to the control. Cellular infiltrations (C) are little. Dilated capillaries between the islets (S) cells are clearly obvious but less than that of Cd treated alone.

Attention was drawn to the role of oxygen radicals and inflammatory mediators in acute pancreatitis [29]. The release of reactive oxygen species in acute pancreatitis might induce autodigestion of acinar cells [30] and pancreatic necrosis which triggers activation of inflammatory cells [31] leading to the production of proinflammatory cytokines, such as interleukin IL 6 [32]. The results of this study confirm and extend the finding of these studies. The current study has displayed a significant increase in the level of MDA a biomarker of lipid peroxidation and an inhibition of SOD involved in antioxidant defense mechanism against free radicals generated following exposure to cadmium in pancreatic tissue homogenate. These results corroborate with Erdogan et al. [33] who observed the same effect of cadmium on MDA and SOD. Also, Olalekan Lawal et al. [34] observed that cadmium induces oxidative stress. It has been documented that Cd-

induced toxic effects are associated with the production of ROS, which can destroy DNA, proteins, and lipid function, and activate signaling pathways that cause cell death [35]. Pancreatic  $\beta$ -cells are at greater risk of apoptosis due to ROS attack than other cell types. The mitochondria of  $\beta$ -cells can generate excessive levels of ROS. They are the major source of ROS in these cells and also a primary target for ROS attack. This, combined with a failure of the ROS defense system, results in the relatively high vulnerability of  $\beta$ -cells to oxidative stress damage [36]. Zhang et al. [37] stated that when SOD and GSH levels drop, the antioxidant capabilities of the pancreas are also reduced.

However, the present study revealed that the increment of MDA and decrement of SOD were normalized with BH<sub>4</sub> administration suggesting that BH<sub>4</sub> may exhibit its preventive effect against cadmium toxicity by enhancing the antioxidant enzyme probably through its free radical scavenging activity. These results concur with the

studies of Ishii et al. [38] who found that BH4 may act as a scavenger of ROS, and may protect  $\beta$ -cells against ROS. Also, Kojima et al. [39] showed that BPH4 inhibited the elevation of lipid peroxides and had extremely strong superoxide anion radical-scavenging activity. Moreover, Vázquez-Vivar et al. [40] explained the antioxidant effects of BH4 in the vasculature by inhibition of superoxide formation from eNOS due to a superoxide scavenging activity of BH4.

The present study showed a significant increased in the level of IL6 and ICAM-1 and disturbed acinar pattern with cellular infiltration between the acini after exposure to cadmium as compared with the control. This observation agrees with Cornet-Boyaka et al. [41] who demonstrated that cadmium treatment induced significant increase in IL-6 and Jiang et al. [42] who reported that Cd increase ICAM-1 expression in renal proximal tubule. Interleukin-6 is an important mediator during inflammatory response, as a part of acute reaction and inducing ICAM-1 expression which regulates neutrophil adhesion [43]. Also, Oxygen free radicals can stimulate the expression of ICAM-1 in the acute pancreatitis and accelerate inflammatory cell infiltration in the pancreas [44]. Zaninovic et al. [45] stated that ICAM-1 is upregulated in pancreas of rats with experimental pancreatitis.

In the current study, investigations emphasized that BH4 treatment significantly decreased the IL6 and ICAM-1 and the acini became more or less similar to the control. These data suggest that BH4 and can effectively suppress the inflammatory damage caused by cadmium in the pancreas. These results concur with Korish and

Arafah [46] who observed that BH4 can decrease the production of the inflammatory markers as C-reactive protein and IL-6. Also, Elio et al. [47] reported that BH<sub>4</sub> treatment attenuates polymorphonuclear neutrophil vascular adherence and tissue infiltration, by inhibiting ICAM-1 expression.

In conclusion, BH4 induced improvements in pancreatic tissue and functions in cadmium-exposed rats. It significantly restores serum amylase, lipase, glucose, insulin levels and ameliorates the disturbed pancreatic tissues. Since cadmium exposure is followed by tissue oxidative stress and inflammation, part of BH4 beneficial effects could be attributed to anti-oxidative and anti-inflammatory activity.

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## الملخص العربي

الأثار الوقائية المحتملة للتيتراهيدروبيوبيترن على تغيرات البنكرياس الناتجة عن الكادميوم في ذكور الجرذان

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يعتبر الكادميوم من الملوثات البيئية والصناعية على نطاق واسع. فإنه يتراكم في البنكرياس ويمكن أن يؤثر على وظائفه. تيتراهيدروبيوبيترن ضروري لمختلف العمليات، وموجود في جميع أنسجة الكائنات الارقى. وقد صممت هذه الدراسة لبحث تأثير تيتراهيدروبيوبيترن على الضرر الحاد للبنكرياس الناجم عن الكادميوم و آلية عمله. تم تقسيم ثلاثين جرذ بشكل عشوائي إلى ثلاث مجموعات (10 جرذان لكل منها). المجموعة الضابطة: اعطيت محلول ملح حقنا داخل البروتون و مجموعة الكادميوم: اعطيت (جرعة واحدة 4 ملجم / كجم كادميوم كلورايد، حقنا داخل البروتون) ومجموعة تيتراهيدروبيوبيترن + الكادميوم: تلقت (جرعة واحدة من تيتراهيدروبيوبيترن 20ملجم / كجم، حقنا داخل البروتون قبل ساعة واحدة من جرعة الكادميوم كلورايد الواحدة 4 ملجم / كجم ، حقنا داخل البروتون). تم قياس مستويات الفا الأميليز، والليباز، والجلوكوز والأنسولين وانترلوكين-6 في مصل الدم وقياس مستويات جزيء الالتصاق بين الخلايا-1، مالونالدهايد و فوق اكسيد الديسميوتاز في كتلة البنكرياس المتجانسة. كما تم عمل فحص نسيجي للبنكرياس. قام تيتراهيدروبيوبيترن بتحسين وظائف البنكرياس، حيث انخفضت مستويات الالفا الأميليز، والليباز و الجلوكوز و انترلوكين-6 انخفاضاً ذو دلالة احصائية بينما زاد مستوى الأنسولين زيادة ذات دلالة احصائية في مصل الدم. قل ضرر البنكرياس كما يتضح من انخفاض مستويات جزيء الالتصاق بين الخلايا-1 و المالونالدهايد وزيادة فوق اكسيد الديسميوتاز في كتلة البنكرياس المتجانسة. أيضا تحسنت أنسجة البنكرياس المتضررة. نستنتج أن تيتراهيدروبيوبيترن أحدث تحسن في أنسجة البنكرياس ووظائفه في الجرذان المعرضة الكادميوم. ويمكن أن يعزى جزء من الأثار المفيدة للتيتراهيدروبيوبيترن لنشاطه كمضاد للأكسدة ومضاد للالتهاب.