World Applied Sciences Journal 21 (5): 681-688, 2013
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2013.21.5.7220

A Possible Inhibitory Effect of Physalis *(Physalis pubescens L.)* On Diabetes in Male Rats

Amal I. Hassan and Mona A.M. Ghoneim

Department of Radioisotopes, Atomic Energy Authority, Giza, Egypt

Abstract: The present study was designed to investigate the possible antidiabetic, hypolipidaemic and antioxidant effects of physalis. Diabetes was induced in Albino rats by administration of alloxan monohydrate (150 mg/kg, body weight i.p). Physalis was administered at a single 1 ml dose per day to these diabetic rats for 21 days. The effect of physalis on blood glucose, serum insulin and pancreatic insulin, serum troponin, tumor necrosis factor (TNFα), interleukin 6 (IL6), vascular endothelial growth factor (VEGF), serotonin, dopamine and malondialdyhyde (MDA) were measured in the diabetic rats. Moreover, on the last day the pancreas were removed and stained with hematoxylin and eosin (H & E) and morphology of the pancreatic sections was studied. Besides, immunohistochemical, insulin and Bcl_2 of pancreas. The results of this study indicate that physalis elicited significant ($p<0.05$) reductions of blood glucose, troponin, TNFα and IL6 except VEGF, dopamine and serotonin were significantly increased. Physalis also caused significant increase in serum insulin ($p<0.05$) in the diabetic rats. The results suggest physalis could be considered as a potential candidate for developing a new anti-diabetic agent. Through, offers promising antidiabetic effects that may be mainly attributed to its potent antioxidant potential. As well as the important factor of this agent in the resistance of beta cells of the pancreas and insulin-producing for the free radicals caused by diabetes. This is the first study on the possibility of *Physalis pubescens* juice as an inhibitory effect on diabetes in rats through its possibility impact of anti-free radicals in the beta cells of the pancreas.

Key words: Diabetes · *Physalis* · Apoptosis · Beta Cells · Neurotransmitter · Tumor necrosis factor · Lipid Peroxidation · Antioxidants

INTRODUCTION

Diabetes arises from a deficient production of insulin. due to cellular mediated autoimmune destruction of pancreatic β-cells of islets of Langerhans and results in loss of insulin production [1] which is essential for the maintenance of normal glucose metabolism, Damage of pancreatic B cells is due to the release of tumor necrosis factor-α (TNF-α and interleukin-1 (IL-1) produced by infiltrating macrophages, lymphocytes and monocytes which leads to the development of type 1 diabetes mellitus (DM) [3, 4]. Alloxan increasing evidence in both experimental and clinical studies suggesting a close relation between hyperglycemia and diabetic complications [5]. It has been widely used to induce diabetes mellitus in experimental animal models allowing investigation of hypoglycaemic agents in the treatment of diabetes [6, 7]. Moreover, it is well known that oxidative stress plays an important role in tissue damage leading to hyperglycemia [5, 7]. Many traditional treatments have been recommended in the alternative system of medicine for treatment of diabetes mellitus; however, the mechanism of most of the herbs used has not been defined [8]. Some plants are well known in traditional herbal medicine for their hypoglycaemic effect. There are more than 800 plant species showing hypoglycaemic activity [9]. Plant products with antidiabetic activity are cheaper, more available and have lesser side effect than medicine. Therefore, plant materials are continuously scrutinized and β cell death causes hyperglycemia due to insulin deficiency, which further aggravates the oxidative stress induced by alloxan [10]. Physalis *pubescens* L. is widely grown in Egypt for at least half a century and is known locally as harankash. It is a member of the

Corresponding Author: Amal Ibrahim Hassan, Department of Radioisotopes, Nuclear Research Centre, Egyptian Atomic Energy Authority , Malaaeb El Gamaa Street , ZIP 12311, Dokki , Giza, Egypt.
E-mail: aml_h@hotmail.com.

Solanaceae family widely used in traditional medicine for the treatment of malaria, asthma, hepatitis, dermatitis and rheumatoid arthritis. Extracts of this plant have showed relevant antioxidant and anti-inflammatory activities [11, 12]. Physalis has long held a place in natural medicine in the tropical countries where it grows and its edible sweet-tart fruits are enjoyed by many rain-forest inhabitants. In recent times Physalis is very much appreciated by great chefs worldwide for their effect as hypoglycaemic agents. Physalis is the most chemical compound with various pharmacological characteristics including, anti bacterial, anti leishmanial and anti tumor and anti-spermatogenesis and anti-conception [13]. The whole plant is anti phlogistic, anti pyretic, anti-tussive and expectorant [14]. It is used in treatment of urinary and skin diseases [15]. Its extract has been used for treatment of wide range of diseases, including kidney and bladder stone, febrile diseases, inflammation, general edema and arthritis [16].

Diabetes mellitus impairs glucose homeostasis causing neurological disorders due to perturbation in utilization of glucose [17]. The present study was conducted to investigate the antidiabetic and monoamines activities of Physalis in alloxan induced diabetic rats. Of unique flavor, lightly acid and sweet, there is no comparison with any other fruit. Furthermore, it is suggested that the therapeutic effect of Physalis on pancreatic damage.

MATERIALS AND METHODS

Chemicals Used: Alloxan monohydrate was purchased from Sigma-Aldrich (MO, IL USA).

Animals: Thirty adult (4-5 month old) male albino rats weighting 200-220 grams were used in the present study. The rats were obtained from the animal house at the Institute of Ophthalmology, Cairo University. All rats had been fed on standardized laboratory balanced diet and given water *ad libitum*. All animals received care in compliance with the national institutes of health criteria for care of laboratory animals.

Induction and Assessment of Diabetes: Diabetes was induced in twenty rats by a single intraperitoneal injection of alloxan (Sigma Chemical Co. St Louis, MO, USA) 150 mg/kg [18]. The animals fasted 12 hours before and after alloxan injection. Alloxan induced diabetic rats with more than 140 mg/dl of blood glucose level were considered to be diabetic and used for the study. Diabetes has been shown after about three days of alloxan injection, the blood samples have been collected via retro-orbital venous plexus and serum glucose levels have been estimated by enzymatic kit method [BioVision USA Cat. No. k 686]. The blood glucose levels have been estimated just prior to killing the three hours fasted-animals at the end of experiment.

Experimental Design: Male rats have been selected at a random way; then have been divided in three groups, each has 10 animals: The first is standard group (non-diabetic animals) G1, the second group (G2) is diabetic control (alloxan-induced diabetic rats) and the third group (G3), are diabetic animals treated with Physalis.

Blood Chemical Analysis: Blood glucose concentration was determined by enzymatic kit method [BioVision USA Cat. No. k 686]. Insulin level was assayed by radioimmunoassay RIA [19]. Quantitative measurements of the Bcl2 protein in the pancreas were made using ELISA [20] with a monoclonal antibody- based kit (BioSource International, Camarillo, CA). Total tissue and cell extracts were lysed with lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM sodium orthovanadate). Cell lysates were obtained from pancreatic tissues and the total protein concentration was determined using the Bradford method [21]. The Bcl2 concentration was expressed as ng/mg tissue protein. Rat IL6, TNF and VEGF were also estimated using an ELISA kit (Life Science Inc. UK). Moreover, dopamine and serotonin were determined by using an ELISA kit (Life Science Inc. UK) [22].

Immunohistochemical Study: Histological sections of pancreas (5μm) were cut from paraffin blocks with a rotary microtome and mounted on poly-l-lysine-coated glass microscope slides. Sections were deparaffinized in xylene, rehydrated in a descending ethanol series (100%, 90% and 70%, vol/vol) and washed in PBS before incubation in 1% (vol/vol) hydrogen peroxide to block endogenous peroxide activity. Immunohistochemical staining was performed in control and diabetic rats. The avidin-biotin peroxidase technique was used as described by Hsu *et al*. [23]. The immunohistochemical reagents used were insulin (Milab) (Malmo, Sweden). Approximately 5μm thin sections were cut. Tissue was dewaxed and dehydrated. Endogenous peroxidase was quenched by immersing in 0.6%H_2O_2 in methanol for 30 min. Primary antibodies were applied and the sections were incubated for 18 h at 4 °C in

moisture chambers. Pre-digestion with 0.4% cin at pH 2.4 for 20 min was done before application of antibodies. Appropriate positive and negative controls were used.

Detection of Insulin Gene by Real-time RT-PCR: Total RNA was isolated from pancreatic tissue homogenates using Trizol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. The RNA samples were then dissolved in RNase-free water and quantified spectrophotometrically. The integrity of the RNA was assessed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, Netherlands) by mixing 2µg total RNA with 0.5µg of oligo$(dT)_{12-18}$ primer in a total volume of 12µL. After the mixture was heated at 70°C for 10 min, a solution containing 50 mmol/L Tris_EHCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5µL RNase inhibitor and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5µL. This mixture was incubated at 42°C for 1 h and then stored at -80°C until further use.

For real time quantitative PCR, 1µl of first-strand cDNA diluted 1:10 in RNase-free water was used in a total volume of 25µl, containing 12.5µl 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 200 ng of each primer. Primers were designed with the Primer Express software package (Applied Biosystems) for the insulin gene (forward 5'-GTACACGTCCCCTGATTTCC-3', reverse 5'- ACACCCA GGACCAGTGTCTC-3') and β-actin gene (forward 5-TGTTGTCCCTGTATGCCTCT-3, reverse 5 TAATGTCA CGCACGATTTCC-3) using sequence information from Gen Bank (accession numbers Mn3022 and J00691, respectively). PCR reaction conditions comprised 95°C for 10 min (1 cycle), 94°C for 15 s and 60°C for 1 min (40 cycles) and amplifications were performed on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism 7900 sequence detection system software (version 2.2) and quantified with the comparative threshold cycle method using β-actin as the housekeeping gene reference control.

Statistical Analysis: Data were expressed as mean ± SE. Differences among means were tested for statistical differences by one way analysis of variance (ANOVA), when differences were significant, Data were statistically analyzed using SPSS version 17.0 (SPSS, Cary, NC, USA). One way analysis of variance (ANOVA) was used to test the variations Duncan's test used for multiple comparisons between groups.

RESULTS

We have noticed that glucose levels significantly increase ($P < 0.05$) in alloxan diabetic rats compared to control ones. Similarly, serum troponin, TNF, IL6 and MDA have been increased due to diabetic conditions in rats. On contrary, the daily administration of phasalis to alloxan-induced diabetic rats reduces: glucose, troponin, TNF, IL6 and MDA. This work shows that the antioxidant properties of physalis neutralize the biological effects of alloxan induced diabetes as well as the oxidative stress due to improper control of blood glucose

Table (1) showed–significant elevation in blood glucose, MDA and troponin levels ($P<0.05$), while the serum insulin and VEGF levels decreased significantly in alloxan diabetic rats compared with normal rats ($P<0.05$). Table (2) showed that administration of physalis tends to bring the parameters significantly towards the normal ($P<0.05$).

In diabetic rats, there is significant changes in dopamine and serotonin were noticed ($P < 0.05$), treatment with 1 ml / rat of juice of physalis modulated these effects if compared to non-treated group ($P<0.05$).

Results revealed that IL6 and TNFα were significantly increased in diabetic rats as compared to control and treated with physalis groups (Table 3). A Bcl-2 protein was measured by ELISA analysis as shown in table (4) diabetic group obviously reduced Bcl-2 expression compared with the control group. Treatment with physalis was associated with greater Bcl-2 relative to the control group.

There was no histopathological alteration observed and the normal histological structure was recorded in (Fig.1). Positive immunological reaction was detected in the island of Langerhans cells (Fig.2). While atrophy was noticed in the island of Langerhans cells in the diabetic groups (Fig.3). The immunohistochemical analysis of pancreatic tissues to detect insulin after 3 days of alloxan treatment show rare immunolabelling (Fig.4). There was no histopathological alteration observed and the normal histological structure was recorded in the rats administered physalis (Fig.5). After one dose of alloxan, rats were simultaneously treated with physalis for 21 days, no pancreatic tissue morphological difference could be seen as compared with normal non-treated rat controls. The immunohistochemical analysis allowed us to assess insulin immunolabelling in the endocrine islets (Fig. 6).

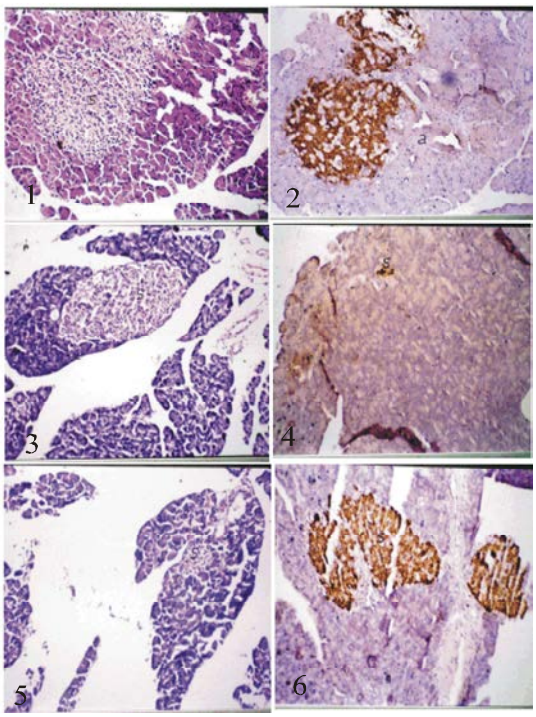

Fig. 1: Control pancreas (H & E X40).
Fig. 2: Immunohistochemical study (control pancreas)
Fig. 3: Section of pancreas in diabetic rats. (H & E X40).
Fig. 4: Immuno histochemical study (pancreas in diabetic rats).
Fig. 5: Section of pancreas in treated rats with physalis (H & E X40).
Fig. 6: Immunohistochemical study (pancreas in treated rats with physalis).

Table 1: Effect of physalis on glucose, insulin, troponin, MDA and VEGF in rats.

Treatment	Control	Diabetes	Treatment with physalis
Glucose (nmol/µl)	$93.70^{b} \pm 2.48$	$167.08^{a} \pm 4.35$	$96.65^{b} \pm 4.58$
Insuline (µU/ml)	$15.75^{a} \pm 1.51$	$9.60^{a} \pm 0.48$	$14.48^{a} \pm 1.54$
Troponin (pg/ml)	$30.75^{c} \pm 1.42$	$45.23^{a} \pm 2.41$	$35.25^{b} \pm 1.80$
MDA (nmol/ml)	$5.25^{c} \pm 0.35$	$8.77^{a} \pm 0.11$	$6.91^{b} \pm 0.19$
VEGF (pg/ml)	$180.70^{a} \pm 1.61$	$145.72^{b} \pm 1.72$	$171.02^{c} \pm 1.33$

Values represent means ±S.E. Values with same superscript in the raw are not statistically different.

Table 2: Effect of physalis on some monoamines in rats.

Treatment	Control	Diabetes	Treatment with physalis
Dopamine(pg/mg)	$82.695^{a} \pm 1.97$	$63.550^{c} \pm 1.53$	$70.780^{b} \pm 2.51$
Serotonin(ng/ml)	$47.270^{a} \pm 1.69$	$26.995^{c} \pm 0.58$	$35.620^{b} \pm 1.41$

Values represent means ±S.E. Values with same superscript in the raw are not statistically different

Table 3: Effect of physalis on IL6 and TNFα in rats.

Treatment	Control	Diabetes	Treatment with physalis
IL6 (pg/mg)	$54.50^{c} \pm 2.18$	$100.43^{a} \pm 4.73$	$66.22^{b} \pm 2.41$
TNFα (pg/mg)	$28.39^{b} \pm 0.90$	$42.17^{a} \pm 1.19$	$31.69^{b} \pm 1.48$

Values represent means ±S.E. Values with same superscript in the raw are not statistically different.

Table 4: Effect of physalis on pancreatic insulin and Bcl2 in rats.

Treatment	Control	Diabetes	Treatment with physalis
Pancreatic insulin (µU/ml)	$1.58^{a} \pm 0.19$	$0.27^{c} \pm 0.05$	$0.89^{b} \pm 0.06$
Bcl2(ng/mg protein)	$3.76^{a} \pm 0.14$	$1.53^{b} \pm 0.03$	$2.87^{ab} \pm 1.06$

Values represent means ±S.E. Values with same superscript in the raw are not statistically different.

DISCUSSION

The pancreas plays an important role in glucose homeostasis [24]. The role of oxidative stress is implicated in the decline of pancreatic function in diabetes mellitus [25, 26]. The possible mechanism for this action might be due to the inhibition of the enzyme glycogen phosphorylase, an enzyme that catalyzes the process of glycogenolysis thereby inhibiting glucagon which on feedback inhibition favours the production of insulin [27]. The diabetic effect of alloxan is due to an excess in the production of reactive oxygen species (ROS). This excess leads to toxicity in pancreatic cells, which, in turn, reduces the synthesis and release of insulin while concurrently affecting other organs, such as liver [28, 29]. The pancreatic β-cells are highly vulnerable to oxidative stress as a result of their intrinsically low expressions and activities of free radical scavenging enzymes [30]. The polyphenols content of the fresh Physalis juice (70mg/100ml) [31, 32]. Physalis polyphenols may, therefore, prevent the damage and death of pancreatic β-cells and/or stimulate the regeneration of this type of cells in diabetic rats. It has been reported that the administration of polyphenols, such as quercetin and epicatechin, to surviving diabetic rats protects the architecture of pancreatic β-cells, preserves the secretion of insulin and stimulates the regeneration of this type of cells [33]. Physalis has been known for a long time in Egypt. Among unexploited tropical fruits, Physalis is a very promising fruit. Recent research has shown cape gooseberry (Physalis *pubescens*) to be high in many beneficial compounds. The antioxidant effect of flavonoids that was found in physalis enhanced the process of regeneration. This might be due to destruction of free radicals, supplying a competitive substrate for unsaturated lipids in the membrane and/or accelerating

the repair mechanism of damaged cell membrane [34]. It is worth noting that the findings indicated that the curative effects achieved with the administration of physalis were pronounced which could presumably be attributed to the large amounts of polyphenols and flavonoids present in physalis [35]. In fact, further studies on the mechanisms and modes of action of physalis are needed to fully appreciate its values and limitations.

Accordingly, maintenance of β-cell oxidant status and their protection against oxidative damage might delay the onset of diabetes as well as the progression of its complications. One of the most often used biomarker to investigate the oxidative damage on lipids is MDA a major lipid peroxidation product. It can react with the free amino group of proteins, phospholipids and nucleic acids leading to structural modification [36]. According to the provided data in Table (1) a notable increase in MDA level was observed in alloxan diabetic rats compared with their respective normal controls. Previous study had reported increased levels of lipid peroxidation in diabetic rats [37]. However, the administration of physalis to the diabetic group of rats significantly reverted back MDA levels to near normal values which show the anti-lipid peroxidative property of physalis in the experimental diabetes. We suggest that the induction of antioxidant enzymatic and non-enzymatic defense systems and suppression of MDA by physalis could be effective in preventing apoptosis activation which might be supported by previous finding [38, 39]. The observed effects of physalis could be related to chemically defined compounds. Flavonoids show their antioxidative action through scavenging or chelating process.

We found a significant elevation of serum inflammatory cytokines (TNFα and IL-6) in our diabetic rat group which was reduced significantly by the oral administration of physalis. TNF-α. IL-1 and IL-6 produced by infiltrating macrophages, lymphocytes and monocytes, damage the pancreatic β -cells and produce type 1 DM by enhancing the formation of oxygen free radicals, lipid peroxides and aldehydes [40].The ability of physalis to reduce the blood glucose level could also be attributed to its ability to modulate the immune system, leading to the decrease of β-cell damages[41]. Physalis anti-inflammatory activity described in this model is related to an immunomodulatory effect exerted on macrophages infected, which directly or indirectly "blocks" their ability to secrete soluble proinflammatory mediators [42].

The current study revealed that alloxan induced significant increased of troponin. The cardiac troponins are biomarkers used for diagnosis of myocardial injury. They are also powerful prognostic markers in many diseases and settings. Recently introduced high-sensitivity assays indicate that chronic cardiac troponin elevations are common in response to cardiovascular (CV) morbidity. Diabetes mellitus confers a high risk of CV disease, but little is known about chronic cardiac troponin elevations in diabetic subjects [43].On the other hand, physalis modulated the elevation of troponin and approached it near normal levels. Ramadan, [36].suggested that goldenberry juice (physalis) has significant hypocholesterolemic activities. Therefore, physalis juice with high antioxidant potential could be helpful for patients suffering from coronary atherosclerosis.

Immunohistochemical study showed that control pancreas showed the typical component of *B*-cells occupying most of the islet cells which produced insulin. At the end of the experiment, diabetic pancreas revealed an almost complete lack of insulin immunoreactivity only a few cells showed a weak and granular staining pattern. Beta cell dysfunction eventually culminates in reduction in insulin release leading to hyperglycemia. The alloxan-induced sustained hyperglycemia aggravates the oxidative stress status by autooxidation of glucose and its primary and secondary adducts [30]. Furthermore, evidence suggests that oxidative stress induced by hyperglycemia may constitute the key and common events in the pathogenesis of different diabetic complications [44].

Immunohistochemical investigation has shown that the immunostaining activity for insulin was decreased in alloxan group. Similarly, decreased of Bcl2 indicating the apoptotic effect of alloxan. The therapy effect of physalis was shown in rats treated with the juice where the levels of Bcl-2 and insulin were increased indicating the anti-apoptotic effect of physalis.

The regulation of apoptosis is another potential mechanism through which many agents such as flavonoids may prevent toxicity. The degeneration observed in the pancreas of alloxan-induced diabetic rats is due to the necrotic action of alloxan monohydrate on the β cells. Earlier work by Bansal, [45] reported specific necrosis of the pancreatic islets after exposure of the islet to alloxan. This degeneration resulted in the inability of the pancreas to secrete adequate insulin for carbohydrate metabolism, which ultimately resulted in the onset of insulin dependent diabetes.

Diabetes is a complex disease associate with peripheral and central complications. These complications include neuropathy. Brain neurotransmitters in this study

were decreased in diabetic rats which may be related to oxidative stress.This finding was confirmed by other studies which indicated that increased brain oxidative stress has been linked to the development of neurodegenerative diseases [46, 47]. Diabetes-induced changes in neural tissues at the presynaptic level, which may underlie alterations in synaptic transmission, particularly if they become permanent during the later stages of the disease [48]. While the improvement of brain neurotransmitters in the treated group may be related to the fact that physalis is an antioxidant that scavenges free radicals directly, inhibit biomolecule oxidation and affects antioxidants *in vivo*. The therapy effects of *physalis pubescens* against alloxan - induced diabetes can be explained on the basis of its nutritional composition. It contains biologically active components e.g. physalins, withanolides, phytosterols and polyunsaturated fatty acids e.g. linoleic acid and oleic acid. Among its major components are high amounts of vitamins A,B and C as well as the presence of essential minerals, magnesium, calcium, potassium, sodium and phosphorus which are classified as macronutrients, while the iron and zinc, are considered as micronutrients [49]. According to Wu *et al*. [50] zinc is a mineral that acts as a nonenzymatic antioxidant, so that its consumption prevents oxidative damage of the cell. Zinc plays an important role in insulin production in the β-cells of islets of Langerhans [51].

CONCLUSIONS

Physalis could be considered as a potential candidate for developing a new anti-diabetic agent. Through, offers promising antidiabetic effects that may be mainly attributed to its potent antioxidant potential. As well as the important factor of this agent in the resistance of beta cells of the pancreas and insulin-producing for the free radicals caused by diabetes. This is the first study on the possibility of *Physalis pubescens* juice as an inhibitory effect on diabetes in rats through its possibility impact of anti-free radicals in the beta cells of the pancreas.

Ethical Guidelines: All animal experiments were performed following the 'Principles of laboratory animal care' National Institutes of Health. (NIH publication No. 85-23, revised 1985), as well as specific local institutional laws for protection of animals under the supervision of authorized investigators.

ACKNOWLEDGMENT

The authors are grateful to Dr. Adel M. Bakeer Kholoussy, professor of, of Pathology, Faculty of Veterinary Medicine, Cairo University for his professional help in carrying out the histopathological examination.

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