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The effect of chloroquine induced hypoglycemia on the levels of major blood serum proteins in diabetic mice

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ABSTRACT

Chloroquine is a drug derived from cinchona bark has been used for long time to treat different diseases including malaria and accidently it was proved to lower hyperglycemia. Diabetes mellitus is accompanied with many disorders including blood serum proteins levels. Taking into consideration that insulin administration controls hyperglycemia of diabetic patients but it is not sufficient to restore the level of blood serum proteins. In the present work we compared the action of chloroquine and insulin on major blood serum proteins of alloxan induced diabetes. Mice were injected once with alloxan and then treated either with chloroquine or insulin. Another category of mice were fed with high glucose diet for short or long period to induce hyperglycemia independent of insulin level. Major blood serum proteins namely; transferrin, albumin, antitrypsin, acid glycoprotein and immunoglobulin G were estimated by SDS-PAGE and Image J software. Our results showed that chloroquine and insulin were independently effective in lowering fasting blood glucose level in alloxan treated animals. Also chloroquine significantly raises blood serum insulin level in diabetic animals without exogenous insulin treatment. Chloroquine restored partially or completely the level of transferrin, antitrypsin, acid glycoprotein and immunoglobulin G significantly and more efficiently than insulin. Both chloroquine and insulin had no influence on restoring the level of albumin in diabetic mice. The results indicate that chloroquine treatment may be a good adjuvant therapy with insulin to control diabetes and its complications.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia, hypoinsulinemia and/or increased cellular resistance to insulin. Type 1 diabetes used to be referred as insulin-dependent DM. It is an autoimmune disease where immune system mistakenly destroys the insulin-secreting β-cells of the pancreas.

Type 2 DM is called non-insulin-dependent diabetes and it is the most common type all over the world.

Exogenous insulin is used for hyperglycemic disorder treatment together with other drugs to facilitate glucose up taking by cells. Among these drugs is chloroquine (cq). Chloroquine is used for the treatment of malaria, rheumatoid arthritis, and collagen vascular disease (Rollo, 1980). Interestingly, there is growing evidence of its beneficial effects on diabetic individuals. The antidiabetic effect of cq was first described in 1984 in a type 2 diabetic patient, where cq dramatically reduced the dose of insulin requirements (Blazar et al.., 1984). Later on, the use of cq or its hydroxylated form hydroxychloroquine is used in experiments and on patients alone or together with other drugs to improve hyperglycemia (Smith et al.., 1987; Quatraro et al.., 1990; Shojania et al.., 1999; Gerstein et al.., 2002; Kang et al.., 2009). Also cq was found to decrease the glycated hemoglobin (HbA1C) in diabetic patients for long term usage (Rekedal et al.., 2010). Diabetes is accompanied with many complications including heart, vascular, kidney, retina and liver disorders. Liver is the main organ responsible for secreting most of blood serum proteins, so the level of these proteins should be influenced by diabetes. Determination of serum protein concentrations has a great value in evaluation of severity and progression of many diseases (Lumiej, 1987). Here we investigated the effect of cq on major blood serum proteins in alloxan-induced hyperglycemia and compared cq effect with insulin treatment and insulin resistant like diabetes induced by high glucose feeding. Our results throw the lights on chloroquine as suggested adjuvant therapy for controlling diabetes and its complications.

MATERIALS AND METHODS
Materials

Alloxan, Glucose, Tritonx-100, Tween-20, all chemicals of Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE), EDTA (ethylene diamine Tetra-acetic acid), EGTA (ethylene glycol Tetra-acetic acid), TMED, PMSF, 2-Mercaptoethanol, and protease inhibitor cocktail were from (Sigma Co. USA). Protein assay kit was purchased from Bio Rad, USA. All other chemicals of high grade- if not mentioned- were purchased from local Suppliers.

Animals and experimental design

Fifty adult male Swiss albino mice weighing 25-30 g. were used in the present work. They were maintained in Assiut University Joint Animal Breeding Unit, faculty of medicine, Assiut University, Assiut, Egypt. All experimental procedures were conducted in strict compliance with the guide of National Institute of Health for the care and use of laboratory animals. Suitable temperature of almost 23 ± 2 °C and 12 hours of light /dark cycle were also taken into consideration. Animals were given free access to standard chow and tap water for one week before experiment for adaptation. Mice were categorized into seven groups as follow. Control group (cnt); fed with normal rodent chow without any treatment. Allox group; injected intraperitoneally with freshly prepared single dose of alloxan monohydrate (180 mg/kg b.w) to induce diabetes. Allox+ins group; diabetic mice were injected daily with recombinant insulin subcutaneously (0.5 iu/kg b.w) for 10 days. Allox+cq group, diabetic mice were given a daily oral dose (50 mg/kg b.w) of chloroquine for 10 days. Cq group, normal untreated mice were orally administrated daily dose of chloroquine (50 mg/kg b.w) for 10 days. S.t.glu group, normal untreated mice orally administered with glucose (0.5 ml of 25% glucose every 6 hours) for 2 weeks. L.t.glu group, where mice orally administered with glucose (0.5 ml of 25% glucose every 6 hours) for 3 month.

Measurement of fasting blood glucose and insulin levels

Fasting blood glucose (fbgl) and insulin levels were measured after overnight fasting. Glucose levels were measured by hand-held glucose test monitor (Lifescan, Johnson and Johnson) from whole tail vein blood and expressed as mg/dl. Serum insulin was quantified using mice insulin ELISA kit (Crystal Chem, USA) according to manufacturer protocol and expressed as pmol/l.

Estimation of major blood serum proteins by SDS-PAGE

Blood were collected from jugular vein during mice sacrifice in non-heparinized sterile tubes. Serum samples were obtained from clotted blood after centrifugation at 5000 rpm for 5 minutes. Total serum protein concentration was estimated by Bio Rad protein assay kit. Twenty micrograms of serum proteins were subjected to 10% SDS-PAG. The gel was stain with Coomassie Blue for 1/2 hour and distained with 40 % methanol and 10 % acetic acid. To confirm the identity of each serum protein, the mobility of each protein was calculated according to Weber and Osborn (1969) and blotted against molecular weight standards expressed on a semi-logarithmic scale. The optical density and the migration distance length of each band were estimated using Image J software.

Statistical analysis

Data were presented as mean ± SD. Statistical analyses were performed using ANOVA. P value < 0.05 was considered significant.

RESULTS

Fasting blood glucose and insulin levels.

The fasting blood glucose level showed significant increase after alloxan administration or fed on glucose when compared with cnt group (Table 1). In contrast, it decreased significantly in allox+ins and allox+cq groups. These results indicate the incidence of hyperglycemia in allox, s.t.glu and l.t.glu groups. Insulin administration greatly restored the level of fbgl near the normal control level. Chloroquine also had the ability to reduce the level of fbgl significantly but less efficiently compared with insulin. Chloroquine alone had no significant effect to restore normal fbgl. Serum insulin was reduced significantly in allox group and restored partially in allox+ins and allox+cq (Table 1). The highest serum insulin level was obtained in s.t.glu group followed by l.t.glu group. The different states of glycemia and insulinemia obtained in this investigation are summarized in Table 2.

Table 1: Fasting blood glucose and insulin levels in different experimental groups..

Group	Fbgl (mg/dl)	Insulin (pmol/l)
cnt	105±5.3	132.4±12.7
allox	462.7±38.6*	48.3±7.6*
allox+ins	124±7.6*,#	97.6±8.5*,#
allox+cq	184.7±2*,#	74.2±6.7*,#
cq	105±5	127±9.3
s.t.glu	209.7±4.6*,#	243.2±22.9*,#
l.t.glu	١81.33±10.2*,#	176.7±21.2*,#

Data are representing the mean ± SD, *, # P< 0.05,* compared with control group and # compared with alloxan treated group.

Table 2: Status description of different groups concerning glycemia and insulinemia.

Group	Description
cnt	Normoglycemic and Normoinsulinemic
allox	Hyperglycemic and Hypoinsulinemic
allox+ ins	Normoglycemic and Normoinsulinemic
allox+cq	Normoglycemic and Hypoinsulinemic
cq	Normoglycemic and Normoinsulinemic
s.t.glu	hyperglycemic and Hyperinsulinemic
l.t.glu	hyperglycemic and Hyperinsulinemic

Major blood serum proteins by SDS-PAGE.

Major blood serum proteins of different experimental groups were separated by SDS-PAGE (Fig. 1). These proteins are; 1- Transferrin (Tf), 2- Albumin (Alb), 3- Antitrypsin (AT), 4- Acid glycoprotein (Agp) and 5- Immunoglubulin G (IgG) light chain. Quantification of each band was carried out using Image J software by calculating optical density and the migration distance length of each band (Fig. 2).
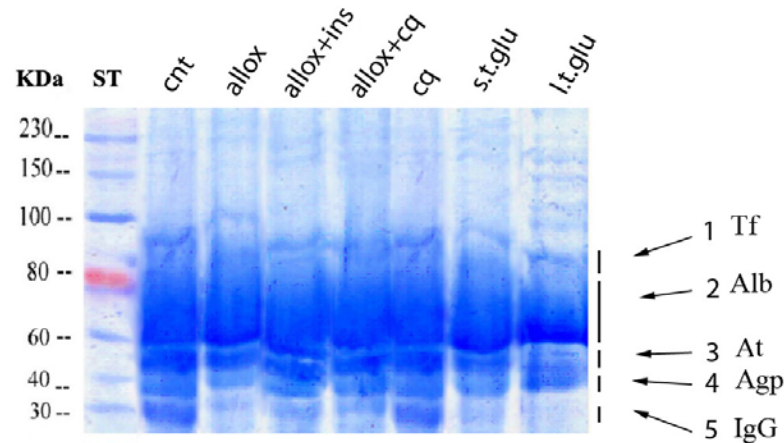

Fig. 1: SDS-PAGE of major blood serum proteins of different experimental groups. Electrophoresis of blood serum and staining with coomassie blue was done as shown in method section. The identity of each protein was carried out by blotting of the mobility of each protein against molecular weight standards (ST) expressed on a semi-logarithmic scale. Band 1 is Tf, Transferrin; 2 Alb, Albumin; 3 AT, Antitrypsin; 4 Agp, Acid glucoprotein and 5 IgG, Immunoglubulin G light chain.
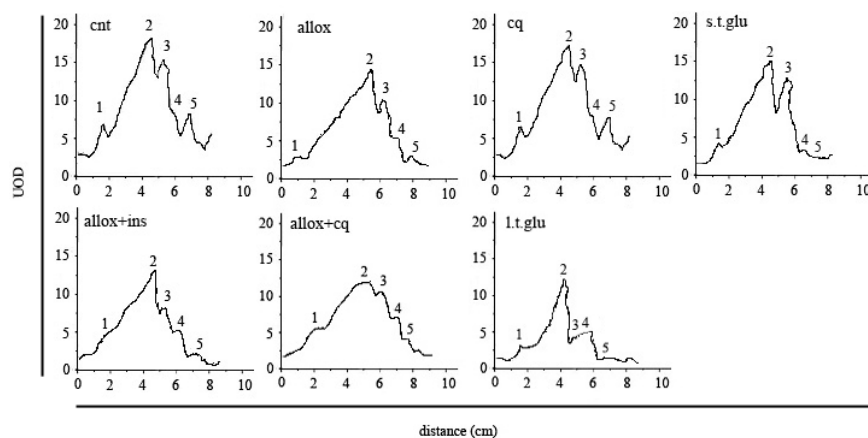

Fig. 2: Calculation of major blood serum proteins in different experimental groups. The SDS-PAGE stained gel was subjected to scan and then the optical density expressed as Uncalibrated Optical Density (UOD) and the migration distance length of each band were calculated by Image J software. Where peak 1 is Tf; 2, Alb; 3, AT; 4, Agp and 5 IgG light chain.

Levels of blood serum transferrin and albumin.

Densitometric quantification of transferrin level in blood serum proteins revealed significant (P<0.05) decrease in its level in allox, s.t.glu and l.t.glu groups when compared with cnt group (Fig. 3, left panel). The mean values were (2.667±0.033,0.8367±0.003,1.207±0.0033, 1.2±0.0057) for cnt and above mentioned groups, respectively.
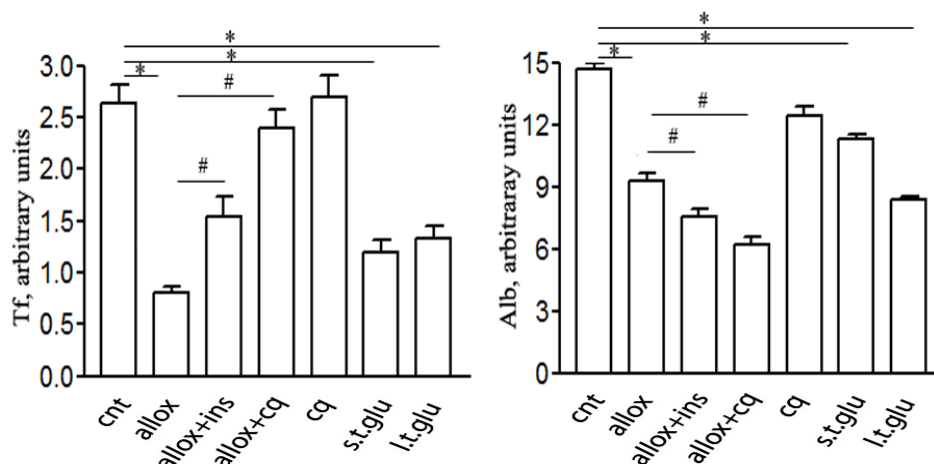

Fig. 3: Tf and Alb levels in different experimental groups. The level of Tf and Alb was calculated from Fig.2 and expressed as arbitrary units. Data are representing the mean ± SD, *, # P < 0.05,* compared with control group and # compared with alloxan treated group.

The level of transferrin significantly (P<0.05) increased in allox+ins and allox+cq groups when compared with allox group. The mean values were (1.623±0.00, 2.43±0.006), respectively. Chloroquine alone did not change the level of transferrin. The level of albumin decreased significantly (P<0.05) in allox, cq, s.t.glu and l.t.glu groups when compared with cnt group (Fig. 3, right panel). The mean values were (14.49±0.05, 9.6±0.06, 10.92±0.06, 7.66±0.03) for cnt and above mentioned groups respectively. Treatments with insulin or chloroquine did not improve the level of albumin where the level of albumin decreased significantly (P<0.05) in allox+ins and allox+cq groups when compared with allox group. The mean values were (7.8±0.06, 6.813±0.06), respectively.

Levels of blood serum antitrypsin, acid glycoprotein and Immunoglobulin G.

Densitometric quantification of antitrypsin level in blood serum protein fractions are shown in (Figs. 4, upper left panel). The level of antitrypsin decreased significantly (P<0.05) in allox, s.t.glu and l.t.glu groups when compared with cnt group. The mean values were (12.41±0.05, 5.5±0.06, 5.4±0.05, 1.57±0.21) for cnt and above mentioned groups, respectively. The level of antitrypsin decreased significantly (P<0.05) in allox+ins group when compared with allox group. The mean value was (3.88±0.04).

This indicates the inability of insulin to restore the basal level of antitrypsin in allox treated mice. But chloroquine partially and significantly (P<0.05) restored the level of antitrypsin (7.58±0.04). The level of acid glycoprotein significantly decreased in allox, s.t.glu and l.t.glu groups when compared with cnt group (Fig. 4, upper right panel).
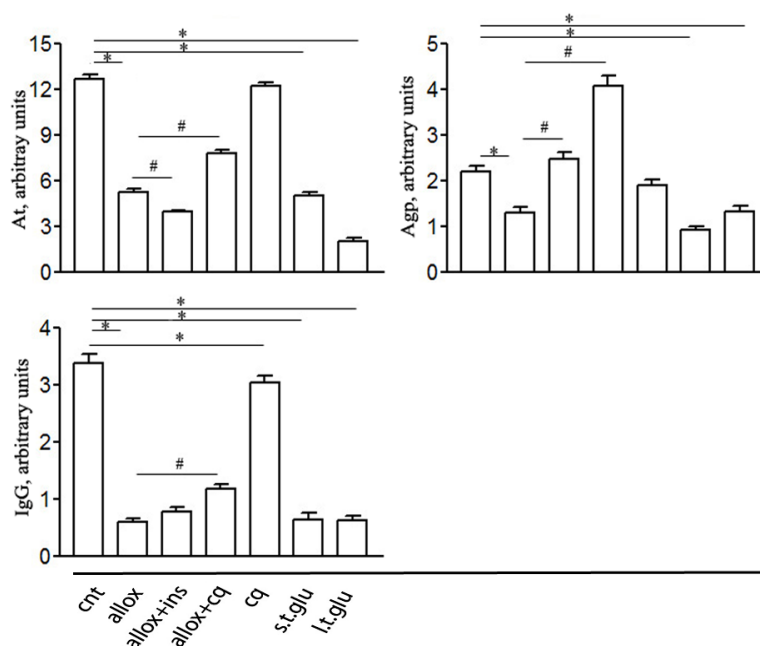
Fig.4: At, Agp and IgG levels in different experimental groups. The levels of At, Agp and IgG were calculated from Fig.2 and expressed as arbitrary units. Data are representing the mean ± SD,*, # P < 0.05,* compared with control group and # compared with alloxan treated group.

The mean values were (2.17±0.09, 1.44±0.02, 0.91±0.037, 1.48±0.046) for cnt and above mentioned groups respectively. Good restoration of the level of acid glycoprotein was achieved upon insulin or chloroquine treatments where its level was (2.4±0.06 and 3.9±0.06) in allox+ins and allox+cq, respectively. Notably, chloroquine alone did not significantly change the level of acid glycoprotein. The level of IgG significantly decreased in allox, cq, s.t.glu and l.t.glu groups when compared with cnt group (fig. 4, lower panel). The mean values were (3.4±0.08, 0.76±0.03, 3.08±0.08, 0.72±0.02, 0.68±0.06) for cnt and above mentioned groups, respectively. Insulin had no significant restoration effect on the level of IgG whereas, chloroquine treatments significantly (P<0.05) had.

DISCUSSION

In the current study, we tried to throw the light on the effect of chloroquine, which is used as adjuvant therapy for diabetes treatment, on the levels of blood serum major proteins.

Alloxan, a β-cytotoxin, is used to induce diabetes in the current study. Alloxan is known to induce chemical diabetes in a wide variety of animal species by damaging β-cells resulting in hyperglycemia (Rajathi and Modilal, 2011and Adeyi et al.., 2012). Ankur and Shahjad, (2012) reported that alloxan induced DNA fragmentation in β-cells which finally stimulates poly ADP-ribosylation for DNA repair. In the current study, the injection of insulin subcutaneously (0.5 IU/kg) daily for 10 days to alloxan induced diabetic mice caused decrease in fasting blood glucose level. Dave and Katyare, (2002) observed that alloxan-induced diabetic condition was confirmed by polyuria, glycosuria, hyperglycemia and loss of body weight, also they observed that insulin treatment regime was able to control these characteristics of diabetes. Here also, the administration of chloroquine orally for 10 days to alloxan induced diabetes caused decrease in fasting blood glucose level. This is in agree with Asamoah et al., (1990) who observed an improved glucose tolerance associated with an

enhanced glucose-induced insulin secretion with chronic chloroquine treatment. Also it was reported that diabetic rats treated with chloroquine for 12 weeks before the onset of diabetes showed significantly higher plasma insulin and protein levels than control diabetic animals (Asamoah *et al.*, 1989), while plasma glucose, glycated plasma protein and glycated haemoglobin levels were lower. It is concluded that after a prolonged administration of chloroquine there is a hypoglycemic effect in normal animals, and pretreatment with the drug ameliorates diabetes induced subsequently. Previous clinical studies made by Powrie *et al.*, (1991) have demonstrated that chloroquine and hydroxychloroquine (HCQ) improve glucose metabolism in patients with insulin-resistant diabetes mellitus but the mechanism of action has not been determined. Ben-zvi *et al.*, (2012) cited that the hypoglycemic effect of chloroquine was first demonstrated in a patient with insulin resistance in 1984. Later on, a clinical trial of non-insulin dependent diabetic patients who were treated with a short course of chloroquine exhibited a significant improvement in glucose tolerance in these patients. When HCQ was combined with insulin for 6 months, the glycated hemoglobin decreased significantly compared to placebo, and the insulin dose had to be reduced by 30% in the HCQ group. The presumed anti-diabetic mechanism of chloroquine is by a decrease in the insulin clearance and degradation rate and an increase in the secretion of C-peptide. The effect of combined administration of insulin and chloroquine on fasting blood glucose in rats placed on diets high in fat and calcium was carried out (Ajani *et al.*, 2004). They concluded that insulin and chloroquine administration resulted in hypoglycemia. Transferrin is the major protein that binds and delivers iron to tissues. Each Tf molecule can transport 2 ferrous iron

molecules to cells by binding to one of the transferrin receptor (TfR) on cell membrane; TfR1 or TfR2 (Namik, 2010). In the current study, SDS-PAGE of serum proteins revealed that the level of transferrin decreased in allox, s.t.glu and l.t.glu groups when compared with cnt group. While allox+ins and allox+cq treatments increased Tf level compared with that in allox group. Low levels of Tf and glycation of amino residues on Tf can enhance the pro-oxidative effects of iron (Van *et al.*, 2004). They argued that these effects were significant causes underlying lipid peroxidation and increase the risk of cardiac vascular disease in diabetes patients. Oxidative damage and neuropathy observed in some diabetes patients lead to increased loss of Tf. Added to this, the glycation of Tf and hemoglobin induced by the higher glucose levels can impair iron binding and promote even higher levels of free iron in the body. Albumin is the single most abundant protein in plasma and accounts for approximately 60% of total plasma protein. The normal liver synthesizes 12–14g/day of albumin, which is rapidly secreted and equilibrated in the intravascular space. In the current study, level of albumin decreased in allox, allox+ins, allox+cq, s.t.glu and l.t.glu groups when compared with cnt group. This result comes in line with (Jelastin *et al.*, 2012) who showed a significant reduction in serum albumin and globulin in alloxan induced diabetic rats. It was suggested that hypoalbuminemia could be divided into four general categories: decreased albumin synthesis, increased albumin loss, redistribution of albumin to locations outside the intravascular space, and dilution of albumin within the intravascular space (Doweiko and Nompleggi 1991). The cause of hypoalbuminemia in a particular patient is often multifactorial. Multiple factors influence albumin synthesis, but clinically relevant decreases in

production are typically due to the following: hepatic failure, inflammation, or chronic malnutrition. Tea *et al.,* (2006) demonstrated that lowering of transferrin and albumin are characteristic changes in inflammation, which may also be part of the pathogenesis of type 2 diabetes. Alpha 1-Antitrypsin (AAT) is one of the major protective proteins in physiological circulation. As a member of the serpin family, AAT inhibits neutrophil elastase, proteinase-3, cathepsin G, thrombin, trypsin, and other proteinases. The protein also has anti-inflammatory properties‹ providing protection from tissue damage in the kidney lung, and liver (Bin *et al.,* 2007). Our study revealed that the level of antitrypsin decreased in allox, s.t.glu and l.t.glu groups when compared with cnt group. This level decreased in allox+ins group and increased in allox+cq group when compared with allox group. Shahaf *et al.,* (2011) cited that AAT is elevated during acute-phase responses and possesses anti-inflammatory properties. For example, AAT increases production of IL-10 and decreases production of IL-6 blocks infiltration of neutrophils and macrophages and reduces nuclear factor (NF)-κB translocation to the nucleus. AAT blocks lipopolysaccharide (LPS) responses in human cells, blocks neutrophil migration and directly binds to IL-8 in lipid rafts. It was cleared that αl-Acid glycoprotein (orosomucoid, OR) is a major glycoprotein of human plasma, and its carbohydrate moiety accounts for 45% of its weight (Michael and Kar 1980). This protein is produced by the liver and is an acute-phase reactant which may has regulatory role of immune responses. There is a strong similarity of amino acid sequences between OR and immunoglobulin. In our study the level of acid glycoprotein decreased in allox, s.t.glu and l.t.glu groups when compared with cnt group. While increased in allox+ins and allox+cq groups when compared with allox group. The current study also showed that the level of immunoglobulin G (IgG) decreased in allox, s.t.glu and l.t.glu groups when compared with cnt group. IgG decreased in allox+ins group and increased in allox+cq group when compared with allox group. IgG levels were significantly lower in the untreated diabetic group, and reduced levels correlated with high values of glycohemoglobin (Hoddinott *et al..,* 1982 and Raphael *et al.,* 2005). This is in contrast to observations of Ardawi *et al..* (1994), who found high levels of IgG in diabetes type 1 and 2 with a positive correlation to glycosylated hemoglobin. They concluded that metabolic control might influence the humoral response and Ig synthesis. It is also possible that impaired intracellular glucose metabolism leads to reduced metabolic activity and consequently, less protein synthesis. Our findings ensure the efficient of chloroquine as an adjuvant therapy for diabetes and its complications. Besides its hypoglycemic effect; it restored most of studied major blood serum proteins. Chloroquine partially or completely restored the levels of transferrin, acid glycoprotein, antitrypsin and immunoglobulin g more efficiently than insulin did. More investigations concerning the use of chloroquine or its hydroxylated form together with insulin in diabetes treatment are still needed.

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ARABIC SUMMARY

تأثير انخفاض مستوى سكر الدم المستحث بعقار الكلوروكوين على مستويات البروتينات الرئيسية لمصل الدم فى الفئران المصابة بداء السكرى

عبد الله بكر محمود- الشيماء ابراهيم الغريانى- ابو بكر محمد الطيب عبد الشكور

معمل بيولوجيا الخلية الجزيئية- قسم علم الحيوان- كلية العلوم- جامعة اسيوط - اسيوط ٧١٥١٦- جمهورية مصر العربية

الكلوروكوين هو عقار مستخلص من لحاء شجرة الكينا استخدم منذ زمن فى علاج العديد من الامراض و اهمها الملاريا و حديثا تم اثبات تأثير هذا العقار على خفض سكر الدم عند الاشخاص المصابون بداء السكرى. داء السكرى يكون مصحوب بالعديد من الاضطرابات وتشمل هذه الاضطرابات حيود بروتينات مصل الدم عن النسب الطبيعية. من الملاحظ ان العلاج بالانسولين لمرضى السكرى لا يكون كافيا لتحسين مستوى بروتينات الدم. فى هذا البحث تم مقارنة تأثير الانسولين و الكلوروكوين كلا على حده على تحسين مستوى بروتينات الدم الرئيسية فى ذكور الفئران المصابة بداء السكرى. تم احداث السكرى المعتمد على الانسولين فى الفئران بواسطة جرعة و حيدة من الالوكسان. مجموعة اخرى من الفئران تم تغذيتها بمحلول سكر الجلكوز المركز لفترة قصيرة او لفترة طويلة لاحداث الاصابة بالسكرى الغير معتمد على الانسولين. تم فصل بروتينات مصل الدم فى كل المجموعات بواسطة الفرز الكهربى للبروتينات باستخدام هلام الصوديوم دودى سلفات و البولى اكريلاميد وبعد ذلك تم التأكد من الوزن الجزيئى للبروتينات بواسطة مقارنتها على منحنى نصف لوغاريتمى لبروتينات معيارية وبعد ذلك تم تقدير كثافتها الضوئية باستخدام برنامج حاسوبى (Image J software). بروتينات مصل الدم الرئيسية التى تم دراستها هى الترانسفرين و الالبيومين و الانتى تربسين و الجليكوبروتين الحامضى و الكرويات المناعية فئة ج. اكدت نتائج الدراسة ان كلا من الكلوروكوين و الانسولين ادى الى خفض مستوى سكر الدم فى الفئران المصابة بالسكرى. الكلوروكوين وحده كان قادر على رفع نسبة الانسولين فى الدم بدون حقن انسولين خارجى. الكلوروكوين كان قادر على تحسين و استعادة مستوى بروتينات الدم (الترانسفرين و الانتى تربسين و الجليكوبروتين الحامضى و الكرويات المناعية فئة ج) بصورة اكثر فاعلية من الانسولين بينما فشل الاخير فى تحسين مستوى معظم هذه البروتينات . كلا من الكلوروكوين و الانسولين لم يكونا قادرين على استعادة او تحسين مستوى الالبيومين. هذه الدراسة دلت على امكانية استخدام عقار الكلوروكوين كعلاج مساعد مع الانسولين لتحسين المضاعفات المصاحبة لداء السكرى.