The effect of insulin induced hypoglycemia on ketoconazole hepatototxicity in rabbit

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ABSTRACT

Background: **Hypoglycemia is one of the most common side effect of insulin treatment, it affect liver and can potentiate ketoconazole toxicity.**

Objectives: **To measure effect of ketoconazole on liver enzymes, hypoglycemic oxidative stress and to evaluate if N-acetylcysteine, can modulate this effect.**

Methods: **Thirty five male rabbits were randomly divided into five groups:**

Group 1: (control group), Group 2:(ketoconazole), Group 3: (insulin), Group 4: (ketoconazole+ insulin), Groups 5: (ketoconazole + insulin + N-Acetyl cysteine). Animals were sacrificed at day 3. Blood collected for measurement of liver enzymes, and total bilirubin. Malondialdehyde and glutathione were measured in serum and liver.

Results: **Ketoconazole increased serum and liver malondialdehyde, 0.594 ± 0.17 and 4614.49 ± 1288.00 nmol/gm. Increased aspartate aminotransferase 38.19 ± 17.29 and alkaline phosphatase 29.29 ± 10.2 U/L. Insulin increased serum malondialdehyde 0.522 ± 0.19, alkaline phosphatase 15.77 ± 6.12 U/L and bilirubin 0.56 ± 0.26 mg/dl. Ketoconazole + insulin, increased serum malondialdehyde 0.850 ± 0.16 µmol/l and bilirubin 0.77 ± 0.55 mg/dl. Ketoconazole + insulin increased serum malondialdehyde 0.850 ± 0.16 µmol/l, aspartate amino transferase 54.35 ± 18.34 U/L, alanine amino transferase, 34.74 ± 11.08 U/L, alkaline pohospahtase 30.81 ± 12.4 U/L and bilirubin 2.51 ± 1.55 mg/dl. N-acetylcysteine reduced aspartate aminotransferase 28.12 ± 22.21 U/L, alkaline phosphatase 11.81 ± 3.03 IU/L) and bilirubin 0.39 ± 0.18 mg/dl**

Conclusion: **Hypoglycemia caused hepatotoxicity and oxidative stress and potentiates the toxicity of ketoconazole. N-acetylcysteine partly reverse this hepatotoxicity.**

Key word: insulin, sitagliptin, Type 2 diabetes

تأثير نقص السكر في الدم الناجم عن الأنسولين على سمية الكبد الناتجة عن الكيتوكونازول في الارانب

خلفية الدراسة: ان نقص السكر في الدم احد الآثار الجانبية الأكثر شيوعا لعلاج الأنسولين في مرض السكري. في حالة خفض الجلوكوز الحاد في الدم، فأن نظام الميتوكوندريا الخاص بإزالة الجذور الحرة يتثبط، الأمر الذي يؤدي إلى توليد أنواع من جذور الأوكسجين التفاعلية. استخدام الكيتوكونازول قد يرتبط بزيادة مخاطر السمية الكبدية. نقص السكر في الدم قد يحفز اصابة الكبد الناجمة عن الكيتوكونازول بواسطة نواتج الأيض من الكيتوكونازول والجذور. عقار NAC كمانح للجلوتاثيون قد يتغلب على الجذور الحرة الناجمة عن نقص السكر في الدم والكيتوكونازول.

الأهداف: قياس وظائف الكبد أثناء نوبات متكررة من نقص السكر في الدم، اختبار تأثير نقص السكر في الدم على تسمم الكبد بفعل الكيتوكونازول، تقييم فعالية الجلوتاثيون على هذه السمية.

الطريقة والمواد: أجريت الدراسة على خمسة وثلاثين من ذكور الأرانب. *قسمت إلى خمس مجموعات*

المجموعة ١: (مجموعة المراقبة)، المجموعة ٢: (الكيتوكونازول)، المجموعة ٣: (الأنسولين)، المجموعة ٤: (الكيتوكونازول + الأنسولين)، *المجموعات ٥:* (الكيتوكونازول + الأنسولين + أسيتيلسياستين). تمت التضحية الأرانب في اليوم الثالث. عينات الدم تم جمعها لقياس مصل (AST، (ALT)، ALP)، ومجموع البيليروبين. تم تقييم حالة الأكسدة بواسطة قياس (MDA) والجلوتاثيون في كل من المصل وجناسة الكبد.

النتائج: المعالجة بمجموعة بالكيتوكونازول أظهرت، زيادة كبيرة في MDA المصل، زيادة كبيرة في MDA الكبد، وزيادة فيAST و ALP، وانخفاض GSH المصل. مجموعة الأنسولين أدت إلى زيادة في MDA المصل، (ALP)، والبيليروبين. عند مقارنة مجموعة الكيتوكونازول مع مجموعة KI، هناك زيادة كبيرة في MDA في المجموعة التي تلقت علاج الكيتوكونازول + الانسولين بالمقارنة مع مجموعة الكيتوكونازول وحدها مع زيادة كبيرة في مستوى البيليروبين. وفيما يتعلق بالمقارنة بين KI والانسولين، كان هناك زيادة كبيرة في MDA في المصل، زيادة في AST, ALT, A والبيليروبين. انخفاض المعلمات التالية في مجموعة KIN بالمقارنة مع KI،(AST) ،(ALP)، (المصل الكليلليبيليروبين).

الاستنتاج: نقص السكر في الدم الناجم عن الانسولين يسبب سمية الكبد والاجهاد التأكسدي وكذلك يؤدي إلى زيادة تحفيز سمية الكبد الناتجة من الكيتوكونازول، ان عقار NAC يمكنه عكس هذا جزئياً.

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INTRODUCTION

Hypoglycemia is a common dangerous side effect of insulin therapy. Failure of glucose supply to the brain causes progressive cognitive impairment, and coma.[1] Hypoglycemia may be defined as a blood glucose level of less than 2.8 mmol /l (50 mg/dl) or the occurrence of classical signs and symptoms.[2] Counter regulatory mechanisms that normally prevent or correct hypoglycemia as the secretion of glucagon, catecholamines, cortisol, and growth hormone[3] are suppressed in diabetics, which makes them frequently prone to the condition.[4] Hepatotoxicity can be caused by exposure to a drug or other chemicals.[5] The liver is the most important site of metabolism and elimination of foreign materials, which make it a target for drug-induced liver injury (DILI) a common clinical problem. DILI is initiated by direct effects of a drug, or its metabolite.[6] Oxidative stress is linked to several diseases including atherosclerosis, neurodegeneration, cancer and diabetes mellitus probably due to the effect of free radicals and reactive metabolites.[7-9] Ketoconazole (KT), an oral antifungal drug used in systemic mycoses, which can occur frequently in diabetics. [10] It was suggested that N-deacetyl ketoconazole, a major and more toxic metabolite, may be responsible for its serious and increasing hepatotoxicity. [11,12] The possible potentiation of hepatotoxic effect of ketoconazole by hypoglycemia will be examined.

MATERIALS AND METHODS

Animal groups

The study was conducted in the Department of Pharmacology, College of Medicine, University of Brash, during the period from September 2013 until December 2014. Animals were divided randomly into five groups (seven rabbits in each group). Blood glucose level was determined at 0, 2, 3 and 4 hrs following each treatment by (Accu-Chek) active glucometer.

Group 1 (Control): Rabbits in this group were treated with 2ml/kg distilled water orally and subcutaneously by the use of insulin syringe at the same time, then 0.5 ml/kg of the solvent dimethyl sulfoxide (DMSO) orally after 2hrs and the animals were sacrificed on the third day of the experiment.

Group 2 (Ketoconazole): Animals in this group were given a single dose of ketoconazole (200mg/kg/day) orally and distilled water subcutaneously at the same time and 0.5 ml/kg DMSO orally after 2hrs for three days, then were sacrificed at the third day of experiment.

Group 3 (Insulin): Animals in this group were given a single dose of insulin (1unit/kg/day subcutaneously) for three days. Other treatments in this group followed the same pattern as in group 1.

Group 4 (ketoconazole + Insulin): Animals in this group were given a single dose of ketoconazole (200mg/kg/day, orally) and insulin (1unit/kg /day subcutaneously) at the same time and 0.5 ml/kg DMSO orally after 2hrs for three days.

Group 5 (ketoconazole + Insulin + Acetylcysteine): Treatments in this group followed the same pattern as in group 4 but they were also treated with a single dose of N-acetyl cysteine (150mg/kg/day orally) two hours after (ketoconazole + insulin) treatment for three days.

Blood sampling

At the morning of the third day of the experiment, the rabbits were anesthetized by inhalation of chloroform and blood samples were collected directly from heart via cardiac puncture instantly before sacrificing the animal. Blood samples, 10ml were collected into non-heparinized tube and allowed for few minutes to clot. Serum was separated by centrifugation. One milliliter of serum was used freshly to measure MDA while the rest of the serum was

frozen at -20 c° for the measurement of other parameters.

Tissue handling

Liver from each rabbit was immediately removed and rinsed in a freshly prepared phosphate-buffered saline, pH = 7.4 . Part of the liver was divided and homogenized for MDA level and liver glutathione measurement. Laboratory measurement of oxidative status in the liver and serum was carried out by thiobarbituric acid reaction. Estimation of liver and serum GSH was done by ELISA method. Kits were used for the determination of liver enzymes, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum total bilirubin.

Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Analysis was made by using SPSS computer package version 15. Independent samples t-test was used to compare between different groups and control. Paired t-test was used to compare differences among the same group. The differences are considered to be significant when $P < 0.05$.

RESULTS

Effect on MDA

The level of serum MDA was significantly increased in all the treatment groups, (Table-1). In multiple comparisons the level of serum MDA was significantly increased in the group treated with (ketoconazole + insulin) in comparison to the ketoconazole and insulin separately (Table-1). The level of liver MDA was significantly increased in the groups treated with ketoconazole and ketoconazole + insulin in comparison to the control group, $P < 0.05$.

Table 1. Effect of different treatments on serum and liver MDA level (mean ± standard deviation)

Treatments	Serum MDA (µmol/l)	Liver MDA level (nmol/gm)
Control	0.300 ± 0.06	2441.13 ± 1317.70
Ketoconazole	0.594 ± 0.17*¥	4614.49 ± 1288.00 *
Insulin	0.522 ± 0.19*¥	3526.41 ± 1230.93 ¥
Ketoconazole+ Insulin	0.850 ± 0.16*	4984.95 ± 1087.49 *
Ketoconazole+ insulin+NAC	0.698 ± 0.29*	3533.39 ± 2152.65

***= Significant from control, ¥ = significant from KI, NAC = N-acetylcysteine**

Effect of treatments on serum aspartate aminotransferase (AST)

The level of AST was significantly increased in the groups treated with ketoconazole and (ketoconazole + insulin) $P < 0.05$ (Table-2). The level of AST was significantly increased in the group treated with the combination of (ketoconazole + insulin) in comparison to the group treated with insulin alone group $P < 0.05$. The level of AST was significantly reduced in the group treated with (ketoconazole + insulin + NAC) in comparison to the group treated with (ketoconazole + insulin), $P < 0.05$.

Effect of treatments on serum alanine aminotransferase (ALT)

Ketoconazole alone, ketoconazole + insulin and ketoconazole + insulin + NAC all caused a statistically P significant increase in serum ALT as compared to the control group. The combination of kectoconazole + insulin caused a statistically significant increase in ALT as

compared to either kectoconazole or insulin alone. The combination treatment (ketoconazole + insulin + NAC) caused a non-significant decrease in the level of (ALT) in the serum in comparison to the combination treatment (ketoconazole + insulin.

Effect of treatments on serum alkaline phosphatase (ALP)

The different treatment groups caused a statistically significant increase in serum ALP levels as compared to the control group. The combination treatment of (ketoconazole + insulin) caused a statically significant < 0.05, increase in the level of (ALP) in comparison to the insulin group. The combination treatment (ketoconazole + insulin + NAC) caused a statistically significant decrease in the level of serum (ALP) in comparison to the combination treatment of (ketoconazole + insulin).

Effect of treatments on serum total bilirubin

The combination treatment (ketoconazole + insulin) cause statically significant, $P < 0.05$ increase in the level of serum total bilirubin in comparison to the control, ketocxonazole and insulin groups. The combination treatment (ketoconazole + insulin + NAC) caused a statistically significant, $P < 0.05$ decrease in the level of Serum total bilirubin in comparison to the combination treatment of (ketoconazole + insulin).

Effect on serum and liver homogenate glutathione (GSH)

There were no statistically significant changes in either the serum or liver homogenate glutathione levels (Table-3).

Table 2. Effect of different treatments on serum AST, ALT and ALP (mean ± standard deviation)

Treatment	Serum AST (U/L)	Serum ALT (U/L)	Serum ALP (U/L)	Serum Bilirubin (mg/dl)
Control	13.51 ± 12.95	11.38 ± 8.76	7.19 ± 2.86	0.31± 0.19
Ketoconazole	38.19 ± 17.29 *	12.47 ± 9.42 ¥	29.29 ± 10.2 *	0.77 ± 0.55 ¥
Insulin	24.06 ± 18.11 ¥	12.66 ±12.00 ¥	15.77 ± 6.12 * ¥	0.56 ± 0.26 ¥
Ketoconazole + Insulin	54.35 ± 18.34 *	34.74 ± 11.08 *	30.81± 12.41 *	2.51± 1.55 *
Ketoconazole + Insulin + NAC	28.12 ± 22.21 ¥	31.67 ± 21.75 *	11.81± 3.03 * ¥	0.39 ± 0.18 ¥

*** = Significant from control, ¥ = Significant from KI**

Table 3. Effect of different treatments on the serum level of GSH (mean ± standard deviation)

Treatment	Serum GSH level (nmol /ml)	GSH level in liver homogenate(nmol / ml)
Control	33.90 ± 21.33	6.7 0 ± 2.63
Ketoconazole	25.22 ± 16.86	5.83 ± 3.71
Insulin	16.63 ± 8.67	6.07 ± 2.87
Ketoconazole + insulin	30.84 ± 15.23	4.74 ± 3.44
Ketoconazole + Insulin+ NAC	20.25 ± 15.33	6.06 ± 1.56

Effect of treatments on blood glucose levels:
Blood glucose levels decreased significantly in the insulin treated group from 107.66 mg/dl at baseline to the level of 55.57 mg/dl, 65.66 mg/dl, and 82.28 mg/dl at 2hrs, 3hrs, 4hrs respectively after insulin injection. Similar changes in blood glucose levels were also occurred in the other insulin containing groups (Table-4).

Table 4. Blood glucose levels (mg/dl) of all groups in different time intervals (mean ± standard deviation)

Treatments	Time			
	0 hrs	2 hrs	3 hrs	4 hrs
Control	104.47 ± 19.54	116.09 ± 36.26 *	115.28 ± 25.96	111.76 ± 24.69
Ketoconazole	102.38 ± 18.26	100.85 ± 26.21	112.19 ± 23.57	103.04 ± 24.20
Insulin	107.66 ± 19.95	55.57 ± 22.66*	65.66 ± 24.38*	82.28 ± 25.35*
KI	106.72 ± 17.77	60.22 ± 20.50*	63.93 ± 28.12*	65.31 ± 25.94*
KIN	109.56 ± 13.69	55.49 ±17.98*	60.26 ± 21.28*	69.67 ± 24.77*

*** = Significant from (0 hrs)**

KI =Ketoconazole + Insulin, KIN = Ketoconazole + Insulin + N-Acetyl cysteine

DISCUSSION

In the present study, administration of insulin (1 unit/ kg) resulted in an insignificant reduction in GSH levels in serum and slight reduction of GSH levels in liver homogenate. This indicates that an oxidative stress state occurs in the hepatocytes, since glutathione is the major biological antioxidant present in the liver. Zhong and Sato[13] found that there is increase of plasma levels of oxidized glutathione (GSSG) and a decrease in the GSH/GSSG ratio and this was associated with increased levels of serum enzymes (AST and ALT) at 1h following the induction of hypoglycemia by intravenous injection of insulin (10 U/kg) in rabbits. MDA levels in serum and liver homogenate increased in the insulin group and this reflects the increasing levels of lipid peroxidation and free radicals generation which may lead to tissue damage, mitochondrial failure, cell death [14] and impairment of endogenous antioxidants defense mechanisms.[15] In the present study the slight elevation of AST and ALT may be attributed to the damaged hepatocytes.[16,17] The liver homogenate MDA was significantly increased in the ketoconazole treated group and this reflects an increase in lipids peroxidation.[18] The mechanism of hepatic injury may be due to ketoconazole or its metabolite N de- acetyl ketoconazole which is more cytotoxic. This toxic metabolite first, damages the smooth endoplasmic reticulum followed the mitochondria and plasma membrane.[19] Ketoconazole treated rabbits had increased levels of MDA in the serum and liver homogenate. Increase levels of transaminases, ALP and total bilirubin occurred to varying degrees. Also ketoconazole (KT) treated rabbits in our study showed slight reduction in GSH levels in liver homogenate and insignificant reduction in serum GSH. This reduction probably related to increased oxidative stress in the liver.[20] The combination treated group ketoconazole + insulin (KI) showed increased levels of MDA in serum and liver homogenate, AST, ALT, ALP and total bilirubin (TBL) and insignificant reduction in GSH level. The mechanism of these increases may be attributed to the augmentation of the oxidative stress processes caused by hypoglycemia and the hepatotoxicity caused by ketoconazole.[21] In the

present study ketoconazole + insulin + NAC treated group showed insignificant reduction in MDA levels in serum and liver homogenate. Also there was insignificant increase in GSH levels in serum and liver homogenate. This may occur in case of hypoglycemia because; due to impaired glutathione synthesis. N-acetylcysteine did not induced complete restoration of GSH levels. This may be due to the unbalance between GSH synthesis and consumption, where the high utilization rate lowers GSH.[22] As regard to the effect of various treatments on glucose levels, the control and ketoconazole groups did not show any significant reduction, in blood glucose levels after 2, 3, 4hrs from baseline, except after 2hrs in the control group where there was a significant increase in glucose which may be attributed to normal physiological changes. All other groups showed a significant reduction in blood glucose levels after insulin injection at the same time intervals. In the (insulin + ketoconazole + NAC) group, the NAC did not correct hypoglycemia. In another study,[23] it was found that the use of NAC lead to prevention of ROS production during hypoglycemia and this was useful in preventing impaired counter-regulatory response in patients undergoing intensive-insulin therapy, probably due to glutathione donation.[24] In conclusion, hypoglycemia lead to an oxidative stress in the liver and derangement of the liver enzymes and this can trigger hepatotoxicity by ketoconazole. Caution should therefore be exerted when prescribing hepatotoxic drugs to diabetic patients.

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