The effect of insulin induced hypoglycemia on ketoconazole hepatotoxicity 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 pohosphatase 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 potentiate the toxicity of ketoconazole. N-acetylcysteine partly reverse this hepatotoxicity.

Key word: insulin, sitagliptin, Type 2 diabetes
INTRODUCTION

Hypoglycemia is a common dangerous side effect of insulin therapy. Failure of glucose supply to the brain causes progressive cognitive impairment, and coma. 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. Counter regulatory mechanisms that normally prevent or correct hypoglycemia as the secretion of glucagon, catecholamines, cortisol, and growth hormone are suppressed in diabetics, which makes them frequently prone to the condition. Hypoglycemia will be caused by exposure to a drug or other chemicals. 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. 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. Ketoconazole (KT), an oral antifungal drug used in systemic mycoses, which can occur frequently in diabetics. It was suggested that N-deacetyl ketoconazole, a major and more toxic metabolite, may be responsible for its serious and increasing hepatotoxicity. 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

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

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

*= 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 ketoconazole + insulin caused a statistically significant increase in ALT as

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.
compared to either ketoconazole 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 statistically 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, ketoconazole 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)

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

* = Significant from control, ¥ = Significant from KI

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum GSH level (nmol /ml)</th>
<th>GSH level in liver homogenate(nmol / ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.90 ± 21.33</td>
<td>6.70 ± 2.63</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>25.22 ± 16.86</td>
<td>5.83 ± 3.71</td>
</tr>
<tr>
<td>Insulin</td>
<td>16.63 ± 8.67</td>
<td>6.07 ± 2.87</td>
</tr>
<tr>
<td>Ketoconazole + insulin</td>
<td>30.84 ± 15.23</td>
<td>4.74 ± 3.44</td>
</tr>
<tr>
<td>Ketoconazole + Insulin+ NAC</td>
<td>20.25 ± 15.33</td>
<td>6.06 ± 1.56</td>
</tr>
</tbody>
</table>
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, and 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)

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

* = 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 serum GSH. 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.

REFERENCES
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