EFFECT OF OZONE THERAPY ON ISCHEMIC REPERFUSION INJURY OF THE HEART AND VASCULAR REACTIVITY IN ADULT MALE DIABETIC ALBINO RAT

By

Adel Shalaby, Safaa Mohamad El-Kotb Saleh*, Sally Said Donia*, and Essam Omar Ibraheim*

Departments of Medical Physiology, Al-Azhar and Menoufiya’ Faculties of Medicine

ABSTRACT

Background: Ozone therapy is a form of complementary medicine treatment that aims to increase the amount of oxygen to the body through the introduction of ozone into the body. Objective: Studying the effects of diabetes mellitus, insulin and ozone on ischemic reperfusion (IR) injury on the heart and vascular reactivity in diabetic rat. Material and Methods: Two hundred and fifty adult male albino rats of local strain, weighing 120-150 ±10 grams each, were used in this investigation and divided into: Group I (Normal control group): 10 rats. Group II (Diabetic group -240 rats) were subdivided into 4 subgroups: Group II-a (Diabetic non-treated group - 40 rats), Group II-b (Diabetic insulin-treated group - 40 rats, and Group II-c (Diabetic ozone-treated group - 80 rats Group II-d (Diabetic ozone and insulin-treated group - 80 rats): Rats were submitted to ozone therapy with concomitant treatment with insulin. Supernatant serum was collected in a dry clean tube for estimation of fasting serum glucose, serum total cholesterol, triglycerides, HDL, LDL, LDH, catalase enzyme, glutathione peroxidase, and SOD. Rat aortic rings preparation were used for estimation of changes in vascular reactivity in response to norepinephrine (10-5), vasopressin (10-6 M), indomethacin (10-6 M) and relaxation of aortic rings (preconstricted by NE (10-5) in response to ACh (10-6) and Na+ nitroprusside (10-6) as estimated in different groups. The organ bath was washed out three times with fresh Krebs' solution before the next substance was added and the rings were allowed to stabilize for 1 hour. All results were presented as the mean ± SEM. The data were analyzed using SPSS program version 12. For comparison of statistical significance between different groups, a one way ANOVA with the post hoc of Tukey's multiple comparison test was used. A value of P ≤ 0.05 was considered statistically significant. Results: Ozone therapy caused significant decrease in fasting glucose, total cholesterol, triglycerides, LDL, and lactate dehydrogenase “LDH”, and significant increase in HDL and myocardial antioxidants (catalase, superoxide dismutase “SOD” and glutathione peroxidase). There were a significant increase in cardiac contractility and heart rate during pre-ischemic and ischemic periods. There was a significant decrease in heart rate accompanied by significant increase in cardiac contractility during reperfusion period. Also, ozone treatment produced a significant decrease in vascular reactivity of aortic rings to norepinephrine, vasopressin and indomethacin, with significant increase in percentage of relaxation to acetyl choline “ACh”. Conclusion: Diabetic complications are attributed to the oxidative stress in the body. Ozone activates the antioxidant system affecting the level of glycemia. Ozone prevents oxidative stress by normalizing the organic peroxide levels by activating superoxide dismutase.

Key words: Diabetes mellitus, insulin, ozone treatment, ischemic reperfusion, vascular reactivity.

INTRODUCTION

In spite of the current optimal therapy, the mortality of patients with ischemic heart disease (IHD) remains high, particularly in cases with diabetes mellitus as a co-morbidity. Myocardial lipid metabolism, inflammation, oxidative
stress, myocardial fibrosis, myocardial apoptosis and mitochondrial damage are considered possible mechanisms for the development and progression of diabetic cardiomyopathy “DCM” (Wang et al., 2011).

Increased production of reactive oxygen species (ROS) seems to be an important biochemical modification in some pathological events that cause complications accompanying diabetes, e.g. atherosclerosis, ischemic heart disease, nephropathy, pulmonary disease, and fatty liver (Baynes, 1991). High glucose level can stimulate free radical production. Weak defence system of the body becomes unable to counteract the enhanced ROS generation. As a result, condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress (Pandey et al., 2010). Certain amount of oxidative stress/ROS is necessary for the normal metabolic processes since ROS play various regulatory roles in cells (Gomes et al., 2012).

Ozone (O₃) therapy can activate the antioxidant system by influencing the level of antioxidant enzymes and some markers of endothelial cell damage (Martínez-Sánchez et al., 2005).

The present work aimed to study the effects of diabetes mellitus, insulin and ozone on ischemic reperfusion injury in the heart and vascular reactivity in streptozotocin (STZ)-induced adult diabetic male rat model.

MATERIALS AND METHODS

Animals:
Two hundred and fifty adult male albino rats of local strain, weighing 120-150 ±10 grams each, were used in this investigation. Rats were kept (each three in cage; 30×30×30 cm) on a standard laboratory diet and water throughout the study period. Rats were divided into:

Group I (Normal control group): 10 rats.
Group II (Diabetic group -240 rats) were subdivided into 4 subgroups as follows:
Group II-a (Diabetic non-treated group - 40 rats).
Group II-b (Diabetic insulin-treated group - 40 rats): Rats were submitted to insulin treatment. Mixtard insulin was injected subcutaneously in a dose of 0.75 IU/100 gm B.W. once daily.
Group II-c (Diabetic ozone-treated group - 80 rats): Rats were submitted to ozone/oxygen mixture through rectal cannulae every day for 15 days at doses of 1 ml/kg. Ozone was produced using a medical ozone generator and used immediately after it has been generated.
Group II-d (Diabetic ozone and insulin-treated group - 80 rats): Rats were submitted to ozone therapy with concomitant treatment with insulin.

Methods:
* After 12 hours fasting, blood samples were collected from the retro-orbital venous plexus of each rat, using a fine heparinized capillary tube introduced into the medial epicanthus of the rat’s eye (Schermer, 1968). Two milliliters of blood were collected in a graduated centrifuge tube, left for clotting at room temperature in a water bath for 15 minutes, and then centrifuged at 3000 r.p.m for 15 minutes. The super-
natant serum was collected in a dry clean tube for:

Estimation of fasting serum glucose (Trinder, 1969).

Estimation of serum total cholesterol and triglycerides (Trinder, 1969).

Estimation of serum high density lipoprotein (HDL - Burstein, 1970).

Estimation of serum low density lipoprotein (LDL - Friedwald et al. (1972).

Estimation of lactate dehydrogenase (LDH) concentration (Scientific Committee, 1982).

Estimation of catalase enzyme activity (Goth, 1991).

Estimation of glutathione peroxidase (GPx) activity (Hafeman et al., 1974).

Estimation of superoxide dismutase activity (SOD - Beauchamp and Fridovich, 1971).

* Hearts from different groups were excised and immediately placed in ice-cold heparinized normal saline. Ascending aorta was cannulated, and retrograde perfusion of the nonworking heart (Langendorff’s method) was initiated with modified Krebs-Henseleit (KH) buffer solution maintained at 37°C. The perfusate was aerated with carbogen and then cut into 2.5–3 millimeter rings. Aortic rings were then suspended in a 10 ml organ bath, containing the freshly prepared Krebs’ solution maintained at 37°C and continuously bubbled with carbogen gas. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph (Shin et al., 2006). Aortic rings were allowed to equilibrate for 60 minutes. A resting tension of 1 g was maintained throughout the experiment. The rat aortic ring preparation was used for estimation of changes in vascular reactivity in response to norepinephrine (10⁻⁵ M), vasopressin (10⁻⁶ M), indomethacin (10⁻⁶ M) and relaxation of aortic rings (preconstricted by NE (10⁻⁵) in response to ACh (10⁻⁶) and Na⁺ nitroprusside (10⁻⁶) as estimated in different groups. The organ bath was washed out three times with fresh Krebs’ solution before the next substance was added and the rings were allowed to stabilize for 1 hour.

* Statistical Analysis: All results were presented as the mean ± SEM. The data were analyzed using SPSS program version 12. For comparison of statistical significance between
different groups, a one way ANOVA with the post hoc of Tukey's multiple comparison test was used. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

The mean value ± SEM of fasting blood glucose of diabetic non-treated rats was 208.9 ±1.6 mg/dl which was significantly higher when compared to the corresponding values in control rats which was 76.5±1.43 mg/dl. That of fasting blood glucose of diabetic insulin-treated rats was 97.4 ± 1.47 mg/dl which was significantly lower when compared to the corresponding values in diabetic non-treated rats, and decreased to be within normal range but still significantly higher when compared to the corresponding values in control rats. In diabetic ozone-treated group, it was 149.1 ± 1.17 mg/dl which was significantly lower from the corresponding values in diabetic non-treated rats, and significantly higher when compared to the corresponding value in control rats. The same value showed an insignificant change from the corresponding values in diabetic insulin-treated and diabetic ozone and insulin treated rats. In diabetic ozone- and insulin-treated group, it was 167.4±1.79 mg/dl which was significantly lower from the corresponding value in diabetic non-treated rats, and significantly higher when compared to the corresponding value in control rats. In diabetic ozone- and insulin-treated group, it was 160.4±1.38 mg/dl which was significantly lower when compared to the corresponding value in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 1).

The mean value ± SEM of fasting serum cholesterol of diabetic non-treated rats was 194.7±2.43 mg/dl which was significantly higher when compared to the corresponding value in control rats which was 71.5±1.02 mg/dl. That of fasting blood glucose of diabetic insulin-treated rats was 84.1±0.95 mg/dl which was significantly lower when compared to the corresponding value in diabetic non-treated rats, and still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 84.9±1.15 mg/dl which was significantly lower when compared to the corresponding value in diabetic non-treated rats, and significantly higher when compared to the corresponding values in control and diabetic ozone- and insulin -treated rats. In diabetic ozone- and insulin-treated group, it was 74.4±1.05 mg/dl which was significantly lower when compared to the corresponding values in diabetic non-
EFFECT OF OZONE THERAPY ON ISCHEMIC REPERFUSION INJURY OF ...

treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 2).

The mean value ± SEM of fasting serum HDL of diabetic non-treated rats was 28.6±0.93 mg/dl which was significantly lower when compared to the corresponding value in control rats which was 44.8±0.74 mg/dl. That of diabetic insulin-treated rats was 32.4±0.88 mg/dl which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats which was 44.4±0.47 mg/dl which was significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 2).

The mean value ± SEM of fasting serum LDL of diabetic non-treated rats was 65.9±1.12 mg/dl which was significantly higher when compared to the corresponding value in control rats which was 43.3±0.88 mg/dl. That of diabetic insulin-treated rats was 51.3±0.79 mg/dl which was significantly lower when compared to the corresponding value in diabetic non-treated rats, and still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 32.2±0.69 mg/dl which was significantly higher from the corresponding value in diabetic non-treated rats, and significantly lower when compared to the corresponding values in control and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group, it was 43.8±0.75 mg/dl which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 2).

The mean value ± SEM of fasting serum LDL of diabetic non-treated rats was 804.9±2.88 U/liter which was significantly higher when compared to the corresponding value in control rats which was 342.8±10.69 U/liter. That of diabetic insulin-treated rats was 603.7±3.1 U/liter which was significantly lower when compared to the corresponding value in diabetic non-treated rats, but still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 700.5±2.05 U/liter which was significantly lower from the corresponding value in diabetic non-treated group, and significantly higher when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone – and insulin-treated rats. In diabetic ozone- and insulin -treated group, it was 352.6±1.53 U/liter which was significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 3).

The mean value ± SEM of catalase of diabetic non-treated rats was 0.273±0.0095U/mg tissue of heart which was significantly lower when compared to the corresponding value in control rats which
was 0.697±0.0093 U/ mg tissue of heart. In diabetic insulin-treated rats, it was 0.494±0.0099 U/ mg tissue of heart which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 0.58±0.0073 U/ mg tissue of heart which was significantly higher from the corresponding values in diabetic non-treated and diabetic insulin-treated group and significantly lower when compared to the corresponding value in control rats. In diabetic ozone- and insulin-treated group, it was 0.66±0.101 U/ mg tissue of heart which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Table 1).

The mean value ± SEM of glutathione peroxidase of diabetic non-treated rats was 0.183±0.0065 U/ mg tissue of heart which was significantly lower when compared to the corresponding values in control rats which was 0.316±0.006 U/ mg tissue of heart. That of glutathione peroxidase of diabetic insulin-treated rats was 0.223±0.0042 U/ mg tissue of heart which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 0.262±0.006 U/ mg tissue of heart which was significantly higher from the corresponding values in diabetic non-treated and diabetic insulin-treated group, and significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Table 1).

The mean value ± SEM of cardiac contractility in stabilization (pre-ischemic) period of diabetic non-treated rats was 1.78±0.043 g tension which was significantly lower when compared to the corresponding value in control rats which
was 3.58±0.071 g tension. That of diabetic insulin-treated rats was 2.58±0.045 g tension which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 2.17±0.041 g tension which was significantly higher from the corresponding value in diabetic non-treated group, and significantly lower when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group, it was 3.45±0.040 g tension which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 4).

The mean value ± SEM of cardiac contractility ischemic period of diabetic non-treated rats was 0.54±0.034 g tension which was significantly lower when compared to the corresponding value in control rats which was 1.74±0.037 g tension. That of cardiac contractility in ischemic period of diabetic insulin-treated rats was 1.23±0.06 g tension which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 2.48±0.059 g tension which was significantly lower when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 1.38±0.052 g tension which was significantly higher when compared to the corresponding value in diabetic non-treated group, and significantly lower when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group, it was 1.59±0.031 g tension which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 4).

The mean value ± SEM of heart rate (beat / minute) in stabilization(pre-ischemic) period of diabetic non-treated rats was 134.7±1.32 beat / minute which was significantly lower when compared to the corresponding value in control rats which was 205.6±1.29 beat / minute. That of diabetic insulin-treated rats was 184.9±1.32 beat / minute which was significantly higher when compared to the
The corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 165±1.15 beat / minute which was significantly higher from the corresponding value in diabetic non-treated, diabetic insulin-treated and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group it was 203.1±1.14 beat / minute which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats. The same value showed an insignificant change from the corresponding value in control rats (Fig. 5).

The mean value ± SEM of heart rate (beat / minute) in ischemic period of diabetic non-treated rats was 7±0.63 beat / minute which was significantly lower when compared to the corresponding value in control rats which was 43.4±0.72 beat / minute. That of diabetic insulin-treated rats was 26.7±0.73 beat / minute which was significantly higher when compared to the corresponding value in diabetic non-treated rats, and still significantly lower when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 15.9±0.48 beat / minute which was significantly higher from the corresponding value in diabetic non-treated group, and significantly lower when compared to the corresponding values in control, diabetic insulin-treated, and diabetic ozone- and insulin-treated rats. In diabetic ozone and insulin -treated group, it was 41.1±0.67 beat / minute which was significantly higher when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 5).

The mean value ± SEM of heart rate (beat / minute) in reperfusion (post-ischemic) period of diabetic non-treated rats was 336±7.29 beat / minute which was significantly higher when compared to the corresponding value in control rats which was 234±5.76 beat / minute. That of diabetic insulin-treated rats was 269.5±2.98 beat / minute which was significantly lower when compared to the corresponding value in diabetic non-treated rats, and still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 291±3.02 beat / minute which was significantly lower from the corresponding value in diabetic non-treated group, and significantly higher when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone-treated rats. In diabetic ozone- and insulin-treated group, it was 249.5±3.36 beat / minute which was significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats (Fig. 5).

The mean value ± SEM of vascular reactivity of aortic strip to 1 x 10⁻⁵ M nor-epinephrine of diabetic non-treated rats was 100.4±1.02 mg tension which was significantly higher when compared to the corresponding value in control rats which was 45.9±1.24 mg tension. That of diabetic insulin-treated rats was 62.4±0.92mg tension which was significantly
lower when compared to the corresponding value in diabetic non-treated rats, but still significantly higher when compared to the corresponding values in control rats. In diabetic ozone-treated group, it was 73.5±1.07 mg tension which was significantly lower from the corresponding value in diabetic non-treated group, and significantly higher when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group, it was 48.9±1.16 mg tension which was significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats. The same value was insignificant with the corresponding values in control rats (Fig. 6).

The mean value ± SEM of vascular reactivity of aortic strip to 1 x 10^-6 M vasopressin of diabetic non-treated rats was 102.4±1.22 mg tension which was significantly higher when compared to the corresponding values in control rats which was 46.1±0.93 mg tension. That of diabetic insulin-treated rats was 64.4±1.24 mg tension which was significantly lower when compared to the corresponding value in diabetic non-treated rats, but still significantly higher when compared to the corresponding value in control rats. In diabetic ozone-treated group, it was 75.3±1.03 mg tension which was significantly lower from the corresponding value in diabetic non-treated group, and significantly higher when compared to the corresponding values in control, diabetic insulin-treated and diabetic ozone- and insulin-treated rats. In diabetic ozone- and insulin-treated group, it was 50.1±1.31 mg tension which was significantly lower when compared to the corresponding values in diabetic non-treated, diabetic insulin-treated and diabetic ozone-treated rats. The same value was insignificant with the corresponding value in control rats (Fig. 6).
Figure (1): Fasting serum glucose (Mean ± S.E.)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal</th>
<th>Diabetic non-treated</th>
<th>Diabetic insulin-treated</th>
<th>Diabetic ozone-treated</th>
<th>Diabetic ozone- &amp; insulin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting serum glucose (mg/dl)</td>
<td>76.5</td>
<td>208.9</td>
<td>97.4</td>
<td>149.1</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Figure (2): Fasting serum cholesterol (mg/dl), triglycerides (mg/dl) LDL (mg/dl) and HDL (mg/dl) in normal, diabetic non-treated, diabetic insulin-treated, diabetic ozone-treated and diabetic ozone- & insulin-treated groups (Mean ± S.E.).

* Significant when compared to the corresponding values in normal group.
• Significant when compared to the corresponding values in diabetic non-treated group.
♦ Significant when compared to the corresponding values in diabetic insulin-treated group.
♥ Significant when compared to the corresponding values in diabetic ozone-treated group.
**Figure (3):** The mean values ± S.E. of LDH (U/liter in the collected perfusion fluid) in control, diabetic non-treated, diabetic insulin-treated, diabetic ozone-treated and diabetic ozone- and insulin-treated rats.

*Significant when compared to the corresponding values in normal group.
• Significant when compared to the corresponding values in diabetic non-treated group.
★ Significant when compared to the corresponding values in diabetic insulin-treated group.
♥ Significant when compared to the corresponding values in diabetic ozone-treated group.

**Table (1):** Mean ± S.E. of catalase, glutathione peroxidase and SOD (U / mg of heart tissue) level in control, diabetic non-treated, diabetic insulin-treated, diabetic ozone- and insulin-treated rats.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catalase (U/mg)</strong></td>
<td>0.697±0.009</td>
<td>0.273±0.0095</td>
<td>0.494±0.0099</td>
<td>0.58±0.0073</td>
<td>0.66±0.101</td>
</tr>
<tr>
<td><strong>P values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DNT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DIT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOIT</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Glutathione peroxidase (U/mg)</strong></td>
<td>0.316±0.006</td>
<td>0.183±0.0065</td>
<td>0.223±0.0042</td>
<td>0.262±0.006</td>
<td>0.302±0.0055</td>
</tr>
<tr>
<td><strong>P values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DNT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DIT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOIT</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Superoxide dismutase activity (U/mg)</strong></td>
<td>50.2±0.663</td>
<td>22.2±0.840</td>
<td>31.4±0.933</td>
<td>38.7±0.578</td>
<td>48.1±0.737</td>
</tr>
<tr>
<td><strong>P values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DNT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DIT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOT</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DOIT</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
**Figure (4):** The Mean values ± S.E. of cardiac contractility (g tension) in stabilization (pre-ischemic), ischemic and reperfusion (post-ischemic) periods in normal, diabetic non-treated, diabetic insulin-treated, diabetic ozone-treated and diabetic ozone- and insulin-treated groups.

* Significant when compared to the corresponding values in normal group (p < 0.05)
● Significant when compared to the corresponding values in diabetic non treated group (p < 0.05)
♦ Significant when compared to the corresponding values in diabetic insulin treated group (p < 0.05)
♥ Significant when compared to the corresponding values in diabetic ozone treated group (p < 0.05)

**Figure (5):** The Mean values ±S.E. of heart rate (beat/minute) in stabilization (pre-ischemic), ischemic and reperfusion (post-ischemic) periods in normal, diabetic non-treated, diabetic insulin-treated, diabetic ozone-treated and diabetic ozone- and insulin-treated groups.

* Significant when compared to the corresponding values in normal group (p < 0.05)
● Significant when compared to the corresponding values in diabetic non treated group (p < 0.05)
♦ Significant when compared to the corresponding values in diabetic insulin treated group (p < 0.05)
♥ Significant when compared to the corresponding values in diabetic ozone treated group (p < 0.05)
DISCUSSION

Clinical studies suggested that diabetic patients have a significantly greater incidence and severity of several cardiopathies (e.g. angina, acute myocardial failure, and atherosclerosis) and approximately 80% of all patients with diabetes die of cardiovascular diseases (Feuvray and Lopaschuk, 1997). Ischemia–reperfusion injury may occur as damage to the myocardium following blood restoration after a critical period of coronary occlusion (Bolli and Marban, 1999). Oxidative stress explains the pathogenesis of ischemia–reperfusion injury (Griendling and Alexander, 1997). Oxidative stress is usually associated with increased formation of reactive oxygen species (ROS), modifies phospholipids and proteins leading to lipid peroxidation and oxidation of thiol groups. These changes are considered to alter membrane permeability and configuration in addition to producing functional modification of various cellular proteins (Suzuki et al., 1997).

Because ozone therapy can activate the antioxidant system, and improve some markers of endothelial cell damage (Martínez-Sánchez et al., 2005), medical ozone treatment could be used as a complementary therapy in the treatment of diabetes and its complications.
Marked fluctuations in glucose levels contribute to more oxidative stress in the diabetic condition, even in patients being treated with insulin (Gao et al., 2012), and with time results in the development of diabetic complications (Ceriello and Testa, 2009). In the present investigation, administration of ozone (1mg/kg B.W.) for 15 days revealed a significant decrease of fasting serum glucose when compared to the corresponding values in diabetic-nontreated rats. This indicated that ozone therapy potentially improved the glycemic control during diabetes. Eman et al. (2013) recorded a significant increase in β-cell number in diabetic ozone-treated group when compared with diabetic non-treated group. Gergorio et al. (2005) observed a significant decrease in the percentage of damaged islets for diabetic rats treated with ozone with regard to STZ group.

In the present study, STZ injected animals exhibited a significantly higher fasting total cholesterol, triglycerides and LDL levels and a significant decrease in fasting serum HDL level when compared to the normal group. These results indicated a significant dyslipidemia in untreated diabetic rats. Sout (2005) considered diabetic dyslipidemia and hyperglycemia to be predictors of cardiovascular complications. The elevation of lipid profiles in STZ-diabetic rats may be attributed to an increase in the rate of lipolysis with a decrease in lipogenesis leading to release more fatty acids into the blood circulation (Agardh et al., 1999). Elevation of serum lipids indicates either the defective removal or overproduction (or both) of one or more lipoproteins (Akula et al., 2003). Diabetic insulin-treated rats exhibited significantly lower fasting total cholesterol, triglycerides and LDL levels, and a significant increase in fasting serum HDL level when compared to the diabetic non-treated group reflecting a partial improvement of dyslipidemia as still there is significant difference when compared to normal rats. As insulin has a profound role in the regulation of key enzymes involved in the lipid and lipoprotein metabolism, its deficiency causes major changes in the activity of these enzymes and thereby affecting overall lipid metabolism and lipid profile of various tissues (Mironava et al., 2000). The altered lipid and lipoprotein pattern observed in diabetic rats could be due to defect in insulin secretion and/or action (Krishnaswami, 1996). Diabetic ozone-treated rats exhibited significantly lower fasting total cholesterol, triglycerides and LDL levels and a significant increase in fasting serum HDL level. Udupa et al. (2012) reported that antioxidants showed an improvement in insulin sensitivity in patients with type 2 diabetes mellitus. So, the improvement of lipid profile with ozone treatment in the present study may be attributed to a relative improvement of insulin level and decreased insulin resistance as a result of controlling the redox state. Haobo et al. (2013) stated that the release of plasma LDH significantly increased in diabetic rats. This indicates that myocardial cellular injury is more severe in diabetic than that in the control rats during reperfusion. Hyperglycemia enhances oxidative stress, and reduces antioxidant defenses (Kain et al., 2011). Compared to the diabetic non-treated group, insulin-treated group exhibited a significantly lower LDH level in the collected perfusion fluid following IR reflecting a
EFFECT OF OZONE THERAPY ON ISCHEMIC REPERFUSION INJURY OF...

Cardio-protective role for insulin. In the rat, insulin reduces the infarct size, plasma creatine kinase and lactate dehydrogenase (both markers of myocardial injury), and apoptosis following IR (Xing et al., 2009).

Ozone-treated group exhibited a significantly lower LDH level in the collected perfusion fluid following IR reflecting a cardio-protective role for ozone treatment on cardiac cell damage after IR. These results were in agreement with Lamiaa et al. (2012) who concluded that ozone therapy can afford significant cardioprotection against biochemical and histological changes associated with IR injury by oxidative preconditioning as it appeared in reducing creatine kinase-MB release, oxidative stress, lactate accumulation, as well as preserving myocardial adenine nucleotides. Histological examination also revealed better improvement with ozone therapy compared to the non-treated I/R group. Filippo et al. (2015) found that ozone treatment led to an evident increase of both systolic and diastolic functions and then of the myocardial performance index with decrease of LDH levels confirming the important role of ozone in the cardio-protection already seen in the present study.

Oxidative stress is the major mechanism that triggers IR injury. Ozone maintains cellular antioxidant systems including glutathione, SOD, and enzymatic reactions, preparing the host to confront the pathophysiologic conditions mediated by oxidative stress (Kesik et al., 2009). The hyperglycemia-induced increase of IRI in diabetes can be prevented by treatment with antioxidants (Akhtar et al., 2012). Several studies revealed that the addition of antioxidants or scavengers, such as SOD and catalase, could reduce infarct size (Chi et al., 1989 and Kilgore et al., 1994). Ozone oxidative preconditioning appears to restore the oxidant balance, minimizing tissue injury caused by IR injury (Bhalla et al., 1999).

Regarding antioxidant enzymes in the present investigation, catalase, glutathione peroxidase and SOD levels in heart tissue partially reversed in insulin-treated diabetic rats when compared to diabetic non-treated rats to a level that was significantly different from control rats, reflecting partial improvement in oxidative stress state with preservation of antioxidant enzymes. Ryuichi et al. (2000) stated that insulin protects cardiomyocytes from oxidative stress-induced apoptosis. Seiichi et al. (2004) indicated that GSH and its related enzyme activities are impaired in diabetic endothelial cells; and these impairments are prevented by treatment with insulin. Insulin can prevent oxidative damage by reducing the formation of peroxynitrite (ONOO) free radicals after myocardial ischemia and reperfusion in the rats (Koo and Vaziri, 2003). In diabetic rat hearts, insulin restores the activity of glutathione peroxidase, a key antioxidant enzyme, correspondingly reduces the toxic process of lipid peroxidation and increases eNOS expression (Zobali et al., 2002).

Regarding antioxidant enzymes in the present investigation, catalase, glutathione peroxidase and SOD levels in heart tissue were partially reversed in ozone-treated diabetic rats. Gregorio et al. (2005) found that blood oxidative stress was controlled by ozone as shown in increased antiox-
dant endogenous systems (superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione). Ozone helps to alleviate oxidative stress associated with diabetes mellitus (Bocci, 2006). The capacity of ozone to enhance antioxidant endogenous systems, in front of oxidative stress by oxidative preconditioning or adaptive mechanisms has been demonstrated (Le?n et al., 1998). Therefore, these results suggest that ozone protective effects on antioxidant endogenous defenses improve glucose metabolism.

In the present study, STZ injected animals exhibited a significantly lower cardiac contractility and heart rate in stabilization (preischemic) period which reflected a significant cardiomyopathy. These results were in agreement with Giuliani et al. (2006), who found that basal cardiac contractility and heart rate decrease in STZ-diabetic rats. Reduction of heart rate is an early event in type 1 diabetes mellitus in a number of species including rats, rabbits, and humans (Wali et al., 2013). Diabetic hearts are chronically subjected to hyperglycemia and hyperlipidemia, both thought to contribute to oxidizing conditions and contractile dysfunction (Niraj et al., 2014). Myocardial triglycerides and cholesterol content significantly increase in diabetic rats (Sharma et al., 2004). Diabetic insulin treated rats exhibited a significantly higher cardiac contractility and heart rate in stabilization (pre-ischemic) period when compared to diabetic non-treated rats reflecting a partial reversal of diabetic cardiomyopathy with insulin treatment and still there is significant difference when compared to normal rats. Giuliani et al. (2006) found that treatment with insulin prevented the occurrence of alterations caused by diabetes, i.e. bradycardia, hypotension and attenuated basal inotropism. The decrease in cardiac contractility induced by chronic diabetes results in part from decrease in expression and alteration in function of rayanodine type 2 calcium release channel in cardiac myocyte, and these changes can be reversed by insulin treatment (Bidasee et al., 2013). Diabetic ozone-treated rats exhibited a significantly higher cardiac contractility and heart rate in stabilization (preischemic) period when compared to diabetic non-treated rats reflecting a partial reversal of diabetic cardiomyopathy with ozone treatment and still there is significant difference when compared to normal rats.

In ischemic period, in the present study, STZ injected animals exhibited a significantly lower cardiac contractility and heart rate when compared to the normal group, reflecting severe decrease in cardiac performance. Jiung-Pang et al. (2009) found that acute myocardial ischemic injury in diabetic rats markedly reduced cardiac output subsequent to bradycardia and reduction of contractility. Ryuko et al. (2007) reported that the hearts of spontaneously diabetic rats were found to be more susceptible to ischemic insult. Cardiac function is critically dependent on substrate utilization, and changes in myocardial fuel selection can have a major impact both positively and negatively (Lopaschuk, 2001). In myocardial ischemic injury, the blood flow in the heart is reduced, decreasing the substrates which are essential to myocardium workload. This pathological condition favors an imbalance between ROS production and the protective antioxidant defense system, thereby
increasing ROS mediated oxidative stress (Hill and Singal, 1996). Moreover, increased oxidative stress plays a critical role in diabetes complications as demonstrated by increased levels of oxidized DNA, proteins and lipids in diabetic subjects (Wiernsperger, 2003). Diabetic insulin-treated rats exhibited a significantly higher cardiac contractility and heart rate when compared to the diabetic non-treated group, reflecting improvement in cardiac performance which still significantly lower when compared to normal rats. The presence of hyperglycemia during myocardial ischemia is closely associated with insulin resistance, which in turn attenuates cardiac sensitivity to exogenous insulin administration (Morisco et al., 2007). In the present study, diabetic ozone-treated rats exhibited a significantly higher cardiac contractility (mg tension) and heart rate (beat/minute), when compared to the diabetic non-treated group, in ischemic period, reflecting improvement in cardiac performance which still significantly lower when compared to normal rats. Ozone therapy leads to the activation of glycolysis with an increase in ATP and 2,3-diphosphoglycerate and increases the release of oxygen in the ischemic tissues (Bocci et al., 2009). So, ozone is used in complementary treatment of hypoxic and ischemic syndromes (Clavo et al., 2003).

In reperfusion (post-ischemic) period, in the present study, STZ injected animals exhibited a significantly lower cardiac contractility and higher heart rate when compared to the normal group, in post-ischemic (reperfusion) period, reflecting development of severe contractile dysfunction and ventricular tachyarrhythmia. Reperfusion of myocardium subjected to a transient ischemia rapidly induces ventricular arrhythmias including VT and VF in both animals and human (Lu et al., 1999). Reperfusion arrhythmias and transient mechanical dysfunction are components of myocardial ischemia-reperfusion injuries (Buja and Weerasinghe, 2010). Oxidative stress caused by reactive oxygen species has a considerable role in ischemia/reperfusion injury, which impairs cardiac function (Inafuku et al., 2013). Diabetic insulin treated rats exhibited a significantly higher cardiac contractility and lower heart rate in reperfusion (post-ischemic) period when compared to diabetic non-treated rats reflecting a partial improvement in recovery of cardiac contractility and decrease in rate of tachyarrhythmia with insulin treatment as still there is significant difference when compared to normal rats. The impairment in functional recovery of diabetic mice after ischemia/reperfusion could be ameliorated by insulin and glucose in perfusates which increased glucose use and enhanced cardiac efficiency (Dragoy et al., 2007).

Diabetic ozone-treated rats exhibited a significantly higher cardiac contractility and lower heart rate in reperfusion (post-ischemic) period when compared to diabetic non-treated rats reflecting a partial improvement in recovery of cardiac contractility and decrease in rate of tachyarrhythmia with ozone treatment as still there is significant difference when compared to normal rats. Ofer et al. (2007) reported a significantly better post-ischemic hemodynamic recovery in ozone-treated rats.
Administration of streptozotocin to rats caused higher vascular reactivity of aortic strips to norepinephrine, vasopressin and indomethacin, significantly lower percentage of relaxation to acetylcholine (endothelium-dependent relaxation), and insignificant changes to Na+ nitroprusside (endothelium-independent relaxation) in aortic strips when compared to the corresponding values in normal rats reflecting endothelial dysfunction and preserved vascular smooth muscles. Naowaboot et al. (2009) reported that vascular responses of the diabetic rats to acetylcholine significantly suppressed, whereas those to phenylephrine significantly increased as compared to normal rats. Wang et al. (2009) concluded that maximum contraction to NA increases significantly in diabetic aorta, and significant decrease in relaxation to ACh in diabetic group compared with controls. Keenoy et al. (2005) also reported a decline of total antioxidant status in diabetes mellitus. It has been shown that vessels from diabetic animals exhibited abnormal endothelium dependent vascular relaxation to acetylcholine. This endothelium-dependent vasodilatation is reduced in diabetes largely due to excessive oxidative stress and decreased bioavailability of nitric oxide (Majithiya et al., 2005). Administration of insulin caused significant decrease in vascular reactivity of aortic strips to norepinephrine, vasopressin and indomethacin and significant increase in percent of relaxation to acetylcholine (endothelium-dependent relaxation) and insignificant changes to Na+nitroprusside (endothelium-independent relaxation) in aortic strips when compared to the corresponding values in diabetic-non treated rats. Lembo et al. (1995) showed that insulin has the ability to modulate the vascular contractile response evoked by various vasoactive substances. The vascular actions of insulin are mediated chiefly through the regulation of endothelium-derived factors. In this regard, insulin can stimulate the production of nitric oxide (NO) (Potenza et al., 2009). Administration of ozone caused significant decrease in vascular reactivity of aortic strips to norepinephrine, vasopressin and indomethacin and significant increase in percent of relaxation to acetylcholine (endothelium-dependent relaxation) and insignificant changes to Na+nitroprusside (endothelium-independent relaxation) in aortic strips when compared to the corresponding values in diabetic-non treated rats. Clavo et al. (2004) showed that ozone therapy could decrease the vasoconstriction. These results were also supported by Al-Dalain et al. (2001), who showed that with ozone treatment there was improvement in aortic relaxation and decreased micro-vessel reactivity, in STZ-experimental model of diabetes. Our results indicated that the combination of insulin treatment with ozone revealed more significant enhancement in oxidative state and improvement in cardiac functions in STZ-induced diabetic rats. According to Sindhu et al. (2004), combined therapy with insulin and antioxidants normalized all measured antioxidant enzyme protein expression and activities. Thus, diabetes-associated reductions in antioxidant enzymes can be ameliorated by combined insulin and antioxidant therapy.

The ozone treatment, by means of its oxidative preconditioning effect, normalizes glucose levels and consequently
restores the concentrations of organic peroxides. So, it contributes to the control of the vascular complications of diabetes.

REFERENCES


56. Scientific Committee (1982): Recommendations for measurement of lactate dehydro-


EFFECT OF OZONE THERAPY ON ISCHEMIC REPERFUSION INJURY OF...

 adel shibli - صفاء محمد القطب صالح - سالى سعيد علي دنيا - عصام عمر إبراهيم

قسم الفسيولوجيا الطبية - كلية طب الأزهر والمنوفية

خلفية البحث: تعتبر أمراض الشريان التاجي من الأسباب المؤدية للوفاة في مرضى السكر. ولذلك فإن العلاجات الدواوية الحديثة يجب أن توجه ليس فقط لضبط مستوي الجلوكوز بالدم ولكن أيضاً إلى منع مضاعفات الجهاز الدوروي. ومحاولة إعادة الإمداد الدموي لعضلة القلب بعد إصابتها باحتشاء عادة ما تكون مصحوبة بنوع من الإصابة أثناء إعادة التغذية الدموية لعضلة القلب حيث تنتج الشفائق الحرة والتي تلعب دوراً مهماً في إحداث عدم التوازن بين المواد المؤكسدة والمواد المضادة للأكسدة. وغاز الأوزون لديه القدرة على زيادة قدرة مضادات الأكسدة.

الهدف من البحث: دراسة تأثير العلاج بالأوزون و/أو الأنسولين على تفاعلية الأوعية الدموية وعلى إصابة عضلة القلب بعد إعادة التغذية الدموية لها في الفئران المصابة بمرض السكري.

مواد وطرق البحث: أجري هذا البحث على 250 من الفئران الذكور البيضاء البالغة التي تتراوح أوزانهم بين 120-150+10 جرام وقد قسم هذا العدد إلى: مجموعة ضابطة غير مصابة بمرض السكر (10 فئران) ومجموعة مصابة بمرض السكر (40 فئران). وقد حق الفئران بمادة الاستربتيوتورين. وقد تم تقسيم المجموعة المصابة بمرض السكر إلى:

- مجموعة مصابة بمرض السكر غير معالجة - مجموعة مصابة بمرض السكر معالجة بالإنسولين - مجموعة مصابة بمرض السكر معالجة بالأوزون - مجموعة مصابة بمرض السكر معالجة بالأوزون والأنسولين.

وفي كل مجموعة من المجموعات السابقة: تم قياس نسبة السكر والدهون بالدم. - قياس قوة إنقباض عضلة القلب قبل وأثناء وقف التغذية الدموية وبعد إعادة التغذية الدموية. - تسجيل عدد نبضات القلب قبل وأثناء وقف التغذية الدموية وبعد إعادة التغذية الدموية. - قياس تفاعليات الأوعية الدموية...
الإنقراضية للأدرنالين والإندوميثاين والفازوربين وكذلك نسبة الأرثاء تحت تأثير الأسبريل كولين ونيتروبروسيد الصوديوم. - قياس إنزيمات مضادات الأكسدة داخل نسيج عضلة القلب. - قياس مستوى إنزيم اللاكتيت ديهدروجينيز الناتج والمرز بواسطة عضلة القلب.

النتائج: لوحظ مع العلاج بالأوزون إنخفاض مستوى السكر والكولستيرول الإجمالي والدهون الثلاثية والكولسترول منخفض الكثافة بالدم وكذلك إنخفاض إنزيم اللاكتيت ديهدروجينيز، كما لوحظ ارتفاع مستوى الكولسترول عالي الكثافة بالدم بعد علاج الفئران بالأوزون، وكانت النتائج ذات دالة إحصائية بالمقارنة مع المجموعة المصابة بمرض البول السكري الغير معالجة، وبحسب أيضا ارتفاع مستوى مضادات الأكسدة داخل نسيج عضلة القلب في هذه المجموعة وكان ذو دالة إحصائية بالمقارنة بالمجموعة المصابة بمرض البول السكري الغير معالجة. كما لوحظ ارتفاع عدد دقات القلب وقوة القلب الإنجابية قبل وأثناء منع التغذية الدموية في هذه المجموعة، وكان ذو دالة إحصائية بالمقارنة بالمجموعة المصابة بمرض البول السكري الغير معالجة. كذلك لوحظ أيضا إنخفاض في عدد دقات القلب مصحوب بارتفاع في قوة القلب الإنجابية بعد إعادة التغذية الدموية في هذه المجموعة وكان ذو دالة إحصائية بالمقارنة بالمجموعة المصابة بمرض البول السكري الغير معالجة. وقد كانت هناك إنخفاضات ذات دالة إحصائية في إستجابة وتفاعلية شريان الأبه للأدرنالين والإندوميثاين والفازوربين، كما كانت هناك ارتفاعات في نسبة الإرثاء وتفاعلية شريان الأبه للأسيتيل كولين في هذه المجموعة، وكانت ذات دالة إحصائية بالمقارنة بالمجموعة المصابة بمرض البول السكري الغير معالجة. كما لم تتغير نتائج إستجابة شريان الأبه لمادة نيتروبروسيد الصوديوم عند مقارنتها بالمجموعة الضابطة.

الاستنتاج: مضاعفات مرض السكر ترجع إلى الإجهاد التأكسدي، والأوزون ينشط مضادات الأكسدة بالجسم.