COMPARATIVE STUDY BETWEEN OXYGEN THERAPY AND RUTIN AS AN ANTIOXIDANT IN HYPERLIPIDEMIC RATS

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ABSTRACT

Obesity is a metabolic disorder and fundamental cause of other fatal diseases including atherosclerosis and cancer. One of the main factor that contributes to the development of obesity is high-fat (HF) consumption. In the present study, the changes in selected biochemical blood variables which are thought to represent risk factors coincident with obesity were compared between a group of normal control male albino rats and other groups suffering from obesity induced by feeding rats on fatty diet (fat 50 % diet). Also, histological studied on the liver were involved. In addition, the effects of two antioxidants (rutin and ozonized water) on the same variables were tested and followed in order to examine to what extent, they are valid to control the levels of these variables without any deleterious effects after treatment. Rutin or ozonized water were daily received orally for one and two months in two groups of obese rats in the following doses 50 mg rutin /kg b.wt/day and 0.5 ml ozonized water /kg b.wt/day, respectively. Fasting blood samples were drawn after one and two months at the terminal of the treatments. The obtained results revealed that induced obesity caused significant (p<0.05) increase of serum leptin, resistin, cholesterol, triglycerides, high density lipoprotein (HDL-Ch), low density lipoprotein-cholesterol (LDL-Ch), very low density lipoprotein-cholesterol (VLDL- Ch) and phospholipids as compared with their relevant level in normal control rats group. On the other hand, induced obesity in rats caused significant (p<0.05) decrease in the levels of serum free triiodothyronine (FT3). No remarkable changes occurred in the concentrations of serum free thyroxin (FT4). Histological examination of liver tissue after HFD feeding showed histological changes illustrated by necrotic cells; Kupffer cells proliferation, fatty degeneration and loss of hepatic architecture. Moreover, all these changes were supported by increase of DNA damage in hepatocytes. A marked correction following treatments of obese rats with rutin or ozonized water.
for one and two months occurred in all previous parameters depending on the time of treatment. The best amelioration occurred in the obese rats group which received the ozonized water at the last interval (60 days). The underlining mechanisms were discussed according to available references.

KEYWORDS: Obesity, Hyperlipidemia, Rutin, Oxygen therapy, Comet assay, Liver.

INTRODUCTION

Obesity is a pathological condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health. It is also the result of an energy imbalance caused by an increased ratio of caloric intake to energy expenditure that it may have an adverse effect on health, leading to reduced life expectancy and/or increased health problems.\(^1\) In fact, the dysregulated energy homeostasis stems from a social reduction in physical activity, energy-dense food combined with a myriad of genetic, social and economic complicating factors.\(^2\)

Obesity is increasing rapidly worldwide among adults as well as among children and adolescents. High fat diet is certainly of importance to obesity incidence and its negative consequences such as hyperlipidemia fatty liver, type II diabetes mellitus, arthritis, lung disease, metabolic syndrome, hypertension, congestive heart failure, urinary incontinence, cataracts, cancer, serious hormonal imbalances, sleeping disorder cardiovascular diseases and aging.\(^3\)

Chronic exposure to high fat diet (HFD) could affect variables at any or all of several levels of control to cause obesity. This could include the taste or other sensory qualities of HF foods, the processing of fat by the gut, the generation and reception of meal-related signals that control food intake and metabolism, the generation and reception by the brain of adiposity-indicating signals and/or brain neurotransmitter systems that regulate food intake and metabolism. Identifying the regulatory processes that mediate HFD induced obesity is of fundamental importance. Increased intake of high caloric (energy and fat) food promotes body fat storage and greater body weight and adiposity in humans.\(^4\) and animals.\(^5\)

Rutin (RT), a quercetin-3-rutinosidand sophorin or vitamin-P, is a well known flavonoidal glycoside and set as an affective phenolic compound. It is an antioxidant, comprised of the flavonolquercetin and the disaccharide rutinose. RT is a phenolic antioxidant and has been demonstrated to scavenge superoxide radicals. It also can chelate metal ions such as ferrous cations.\(^6\) Various pharmacological
properties were reported for RT including antibacterial, antitumor, anti-inflammatory, anti-diarrheal, anti-ulcer, anti-mutagenic, vasodilator, immunomodulator, anti-hyperlipidemia, myocardial protecting and hepatoprotective activities.[7] Moreover, RT has inhibitory effects against membrane lipid peroxidation and generation of ROS.[8] and can suppress adipocytes differentiation from pre-adipocytes.[9] In addition, RT can also decrease the level of TBARS and increase the SOD activity suggesting a possible protective role in oxidative stress-mediated diseases.[10]

Ozone (O₃), a gas discovered in the mid-nineteenth century, is a molecule consisting of three atoms of oxygen. In medical use the gas produced from medical grade oxygen is administered in precise therapeutic doses and never via inhalation. It causes decreased blood cholesterol, stimulation of antioxidative responses, modifies oxygenation in resting muscle and is used in complementary treatment of hypoxic and ischemic syndromes.[11]

So, the present study was undertaken to investigate the complication of obesity on hormonal profile, lipid profile, hepatic phosholipid, total antioxidant capacity, glutathione and lipid peroxidation in addition, the work evaluate the role of oxygen therapy and rutin as an antioxidant to overcome such complications.

MATERIAL AND METHODS

Chemical sources

Rutin

Rutin in the form of rutin hydrate was purchased from Sigma Company, USA. It was suspended in distilled water and freshly prepared just before the administration.

Ozonized water

O₃ gas is allowed to pass through 1 liter bidistilled water (aqua bidestillata). In bidistilled water, the half life of ozone at room temperature is about 10 hours. Water was reozonized once every morning. In a refrigerator, ozonized aqua bidistilled keeps for about 5 days.

Experimental animals and diet

Seventy adult male albino rats from the Serum and Antigen Laboratories at Helwan were employed in the present study. Their weights ranged between 150-170g representing an age group of 7-8 weeks of age. They were housed in a well ventilated ventilated vivarium of Zoology Department, Women's College, Ain Shams University. Animals were allowed a one week pre-experimentation period and were maintained under standard housing conditions.
Fresh supplies of food and water were daily presented. The control and high saturated fat diet composition are illustrated in (Table1).

**Table (1): Composition of the normal and fat diets**

Diets were prepared according to the methods of Moraes et al.\(^\text{[12]}\)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Control diet</th>
<th>HFD(Fat 50 % diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>51.55 %</td>
<td>9.55 %</td>
</tr>
<tr>
<td>Soya bean</td>
<td>18.15 %</td>
<td>18.15 %</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.93 %</td>
<td>10.13 %</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.15 %</td>
<td>5.15 %</td>
</tr>
<tr>
<td>Cellulose</td>
<td>5.15 %</td>
<td>5.15 %</td>
</tr>
<tr>
<td>Vitamin &amp; mineral mixture</td>
<td>2.07 %</td>
<td>2.07 %</td>
</tr>
<tr>
<td>Lard</td>
<td>zero %</td>
<td>50.00 %</td>
</tr>
</tbody>
</table>

**Experimental design**

In the first part of the experiment rats were randomly divided into two main groups of 35 rats each fed on either standard diet (control group) or 50% fat diet (obese group) for six weeks. In the second part of the experiment HFD stopped where main group one animals were further assigned to 3 subgroups of 10 rats each. The first was left untreated while subgroup 2 was orally treated with rutin (50 mg/kg b.wt,\(^\text{[13]}\)) and subgroup 3 orally treated with ozonized water (0.5 ml/100 g,\(^\text{[14]}\)). Main group two was also further assigned into three subgroups of 10 rats each: recovery obese subgroup; obese subgroup treated with rutin (50 mg/kg b.wt) and obese subgroup treated with ozonized water (0.5 ml/100 g).

**METHODS**

**Sample collection and Histological studies**

Five rats from each main group (after 6 weeks) and subgroup were selected after 30 and 60 days of experimental duration and weighed. Autopsies were performed by ether inhalation anesthesia and blood samples were collected from the heart in clean dry test tubes. Animals were sacrificed and liver was rapidly dissected, thoroughly washed with isotonic saline and blotted dry. Liver samples were divided into two halves, one half immediately homogenized for biochemical analyses and the other half fixed in formalin for histological investigation. Where, sections of 6µm thickness were prepared. For general histopathological examination, sections were stained by haematoxylin and eosin.

**Biochemical analysis**

Serum free triiodothyronine (FT\(_3\)) and free thyroxin (FT\(_4\)) levels were estimated by a radioimmunoassay method kit using solid phase component system according to the method of
Siegel et al.\textsuperscript{[15]} and Ekins,\textsuperscript{[16]} respectively. The kits were purchased from Diagnostic Product Corporation (DPC) USA. The concentration of serum resistin and leptin was assayed by ELISA (Sandwich Immunoassay Technique) using commercial kits (IBL-Hamburg, Co. Germany) according to Maffei et al.\textsuperscript{[17]} Serum total cholesterol, triglycerides and HDL-cholesterol were estimated enzymatically using commercial kits from Randox, Ltd., Co. (UK) according to the method of Sidel et al.\textsuperscript{[18]} Fossati and Prencip,\textsuperscript{[19]} and Stein,\textsuperscript{[20]} respectively. LDL-cholesterol and VLDL–cholesterol was calculated as per Freidewald’s,\textsuperscript{[21]} equation: LDL-Chol. = Total Chol. – [TG/5 – HDL- Chol.] and VLDL= TG/5. Phospholipids were determined using commercial kits obtained from Cell Biolabs’ OxiSelect\textsuperscript{TM}, according to the procedure of King and Woolton.\textsuperscript{[22]} Total antioxidant capacity (TAC) was determined using commercial kits obtained from Cell Biolabs’ OxiSelect\textsuperscript{TM}, according to the method described by Allard.\textsuperscript{[23]} Glutathione (GSH) was determined using commercial kits obtained from Cell Biolabs’ OxiSelect\textsuperscript{TM}, according to the procedure of Halliwell and Gutteridge.\textsuperscript{[24]} Lipid peroxidation was evaluated by colorimetric method using commercial kits according to the procedure of Ohkawa et al.\textsuperscript{[25]}

**Detection of DNA damage by the comet assay**

The portion of the liver was minced and suspended in chilled homogenizing buffer (pH 7.5) 0.075µ NaCl and 0.024µ Na\textsubscript{2}EDTA and the homogenized gently using homogenizer in ice. The cells suspension was centrifuged at 4\textdegree C, 700\times 9 for 10 min. The cells were resuspended in the cold buffer.\textsuperscript{[26]}

The comet assay was carried out under alkaline conditions, basically as described by Singh et al.\textsuperscript{[27]} Slides were stained with 50 A of ethidium bromide (2 mg/ml) and observed at 400 magnifications using a lexica DFC 425 camera.

Analysis of comet parameters was performed using TriTek comet score, version 1.5. The DNA damage was quantified by measuring the displacement between the genetic material of nucleus (comet head) and the resulting (tail). The number of tailed cells and tail DNA % are the two most commonly used parameters to analyze the result of the comet assay.\textsuperscript{[28]}

**Statistical analysis**

In the present study, Results were expressed as Mean + S.E of the mean. Data were analyzed by one way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 10. The level of significance was set at P value > 0.05.\textsuperscript{[29]}
RESULTS

Total body weight

The obtained data of the total body weight are given by table (2). Normal rats showed more or less constant levels during the course of the study. Moreover, no remarkable changes were reported after rats were treated with either rutin or ozonized water throughout the experimental duration.

From table (2) in the obese rats group which was fed HFD, a significant elevation in the body weight was recorded. The percentage of increment was 93.50 at the first interval (30 days) as compared to their corresponding animals in the normal control, while this percent of elevation in body weight reached 93.89 at the last interval (60 days). Additionally, the supplementation of rutin to obese rats group led to a significant (p<0.05) decrease in the body weight but, the maximum correction in the body weight was recorded in the obese rats group which were treated with ozonized water and this correction depended on the time of supplementation where the percentage of changes were -0.49% and -5.70% respectively. (table2).

Table (2): Amelioration effect of rutin and ozonized water on the total body weight in obese male rats at various time intervals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Control rutin</th>
<th>Control ozonized water</th>
<th>Recovery Obese group</th>
<th>Rutin</th>
<th>Ozonized water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>179.9±2.56</td>
<td>188.9±1.39</td>
<td>189.4±0.39</td>
<td>300.7±2.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 days</td>
<td>186.3±2.81</td>
<td>189.4±1.66</td>
<td>360.5±1.91</td>
<td>276±2.18</td>
<td>260.8±2.67</td>
<td></td>
</tr>
<tr>
<td>60 days</td>
<td>201.5±2.39</td>
<td>201.9±2.71</td>
<td>360.5±2.17</td>
<td>200.5±2.24</td>
<td>190±2.6</td>
<td></td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE.
- A,B,C,D Means with a common superscript within a row are not significantly different (P>0.05).
- a,b,c Means with a common subscript within a column are not significantly different (P>0.05).

Histological investigation of liver tissue

Sections of livers of animals from the control group rats after oral daily treatment with rutin (50 mg/ kg b.wt) and rats given ozonized water at a dose 0.5 ml revealed a normal hepatic...
configuration. The liver cells are arranged in the form of cords radiating from central vein to the periphery (Fig. a, b & c).

Examination of the liver sections of obesity rats (after 6 weeks fed HFD) revealed inflammatory cell infiltration in portal areas and in between hepatocytes with hemorrhagic changes observed (Fig.d).

Section of liver from recovery subgroup after 60 days showed fat vacuoles and cytoplasmic vacuolation of most of hepatocytes with loss of hepatocytic architecture (Fig.e).

Microscopical changes of liver of obesity rats treated with rutin (50 mg∕kg b.wt) for 30 days were characterized by early variable grades of degenerative changes in hepatic parenchyma in different areas of the liver tissue (Fig.f). At the end of this investigation, (i.e.60 days) post treatment within rutin limited fatty degenerative change with dilated sinusoid and necrotic hepatocytes seen (Fig.g). Liver section from obesity rat group treated with ozonized water after 30th day showed infiltrative inflammatory lymphocytic cells and dilatated portal vein (Fig.h). By the 60th day post experimentation hepatic structure appeared near to normal (Fig.j).
Fig. (a): Section of liver tissue section of rat in the control group showing normal histological structure of the centre vein (cv) and surrounding hepatocytes (h). Fig. (b): Section of liver from control rat treated with rutin after 60\textsuperscript{th} day to show general structure. Fig. (c): Section of liver from control rat treated with ozonized water to show normal configuration of hepatic tissue.
Fig. (d): Liver section from obesity rats at zero time showing inflammatory cells infiltration (m) detected in the portal area and in between the hepatocytes ( ) with hemorrhagic change. Fig. (e): sections of liver from obesity rat group after 60th day showing fat vacuoles and cytoplasmic vacuolation of most of hepatocytes and loss of hepatic architecture. Fig. (f): Section of liver from obesity rat group treated with rutin (50mg/kg b.wt) after 30th day showing fatty change of hepatocytes ( ). Fig (g): Liver section of obesity rat treated with rutin (50 mg/kg b.wt) rat after 60th day showing limited fatty degenerative change, dilated sinisual space and necrotic hepatocytes. Fig (h): Liver section from obesity rat group treated with ozonized water after 30th day showing infiltrative inflammatory lymphocytic cells and dilatated portal vein (PV). Fig (i): Section of liver from obesity group rats treated with ozonized water 60th day to show general structure.

Biochemical studies

In the first experimental, the obtained data in table (3) clarified a significant (P<0.05) elevation in serum cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein and hepatic phospholipids levels in obesity rats. The percentage change recorded 94.95%, 98.23%, 30.1%, 139.17%, 98.31% and 51.76% respectively for cholesterol, triglycerides, HDL, LDL, VLDL and hepatic phospholipids in obesity rats compared to normal control rats. On detecting serum free triiodothyronine (FT₃) level using the data tabulated in table (3). It is recognized that rats fed on HFD revealed a significant (P<0.05) decrease in FT₃ level and the percentage of the change was -42.97% as compared to the corresponding control rats. While, no remarkable a change was occurred in the level of serum free thyroxin (FT₄) in both control rats and obesity rats group and the percentage of change was 1.83%. Moreover, a significant (P<0.05) elevation in the serum resistin and leptin level was noted in obesity rats group as a result of feeding the animals on...
HFD and the percentage of these changes were 179.08% and 68.43% respectively. The levels of total antioxidant capacity (TAC) and glutathione (GSH) were significantly (P<0.05) decreased in obesity rats where, the percentage of these changes were -63.05 and -114.47%. But, the levels lipid peroxidation were significantly (P<0.05) increased in obesity rats and the percentage change of this was 119.51%.

Table (3): Comparison between control and obesity rats in some parameters related to hyperlipidemia.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control rats</th>
<th>Obesity rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT3 (pg/ml)</td>
<td>1.21±0.073</td>
<td>0.69±0.034</td>
</tr>
<tr>
<td>FT4 (ng/dL)</td>
<td>0.601±0.020</td>
<td>0.590±0.025</td>
</tr>
<tr>
<td>Resistin (ng/ml)</td>
<td>1.53±0.051</td>
<td>4.27±0.071</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>4.91±0.081</td>
<td>8.27±0.169</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>60.27±1.31</td>
<td>117.50±1.89</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>68.17±1.39</td>
<td>135.14±1.94</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>19.33±0.59</td>
<td>25.15±0.54</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>27.31±0.54</td>
<td>65.32±0.78</td>
</tr>
<tr>
<td>VLDL (mg/dl)</td>
<td>13.63±0.27</td>
<td>27.03±0.57</td>
</tr>
<tr>
<td>Hepatic phospholipids (mg/g)</td>
<td>30.52±3.21</td>
<td>46.32±5.10</td>
</tr>
<tr>
<td>TAC (mg/g)</td>
<td>6.09±0.14</td>
<td>2.25±0.42</td>
</tr>
<tr>
<td>GSH (mg/g)</td>
<td>14.82±1.05</td>
<td>6.91±0.85</td>
</tr>
<tr>
<td>MDA (mg/g)</td>
<td>3.74±0.12</td>
<td>8.21±0.47</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE
- A, B Means with a common superscript within a row are not significantly different (P>0.05).
- a Means with a common subscript within a column are not significantly different (P>0.05).

In the experimental two, a significant (P<0.05) depletion was occurred in the levels of serum cholesterol, triglycerides and low density lipoprotein (LDL), very low density lipoprotein and hepatic phospholipids levels in obesity rats after treatment with rutin (50mg/kg b.wt/day) for two months (Table 4). The maximum correction effect was reported in the obesity rats which treated with ozonized water dependent on the time of treatment (30 & 60 days).

Table (4): Amelioration effect of rutin and ozonized water on lipid profile and hepatic phospholipids in obese male rats at various time intervals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Non-obesity normal groups</th>
<th>Obesity groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>30 days</td>
<td>62.53</td>
<td>60.51</td>
</tr>
</tbody>
</table>

- A, B Means with a common superscript within a row are not significantly different (P>0.05).
- a Means with a common subscript within a column are not significantly different (P>0.05).
<table>
<thead>
<tr>
<th>(mg / dL)</th>
<th>60 days</th>
<th>30 days</th>
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<th>30 days</th>
<th>60 days</th>
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<tbody>
<tr>
<td>Triglycerides</td>
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<td></td>
</tr>
<tr>
<td>60 days</td>
<td>60.42±1.33</td>
<td>69.32±1.34</td>
<td>69.21±1.34</td>
<td>20.21±0.62</td>
<td>19.36±0.58</td>
<td>28.46±0.57</td>
<td>27.22±0.55</td>
<td>13.86±0.29</td>
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<td>30 days</td>
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<td>LDL - Cholesterol</td>
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<td></td>
<td></td>
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<tr>
<td>60 days</td>
<td>61.91±1.28</td>
<td>67.81±1.30</td>
<td>67.10±1.18</td>
<td>20.52±0.50</td>
<td>19.10±0.51</td>
<td>28.43±0.52</td>
<td>28.84±0.49</td>
<td>13.56±0.26</td>
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<tr>
<td>30 days</td>
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<td>HDL - Cholesterol</td>
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<tr>
<td>60 days</td>
<td>62.10±2.78</td>
<td>66.71±3.76</td>
<td>68.31±1.29</td>
<td>28.54±0.53</td>
<td>19.60±1.24</td>
<td>26.03±8.53</td>
<td>28.84±5.95</td>
<td>13.34±0.26</td>
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<tr>
<td>30 days</td>
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<tr>
<td>Hepatic phospholipids</td>
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<td>(mg / dL)</td>
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</tr>
<tr>
<td>60 days</td>
<td>30.61±1.31</td>
<td>30.75±1.32</td>
<td>30.50±1.54</td>
<td>30.50±2.11</td>
<td>30.31±0.62</td>
<td>30.61±1.34</td>
<td>30.50±1.54</td>
<td>30.31±0.62</td>
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<tr>
<td>30 days</td>
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</tbody>
</table>

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- A,B,C,D Means with a common superscript within a row are not significantly different (P>0.05).
- a,b Means with a common subscript within a column are not significantly different (P>0.05).

From table (5), a considerable improvement was occurred in the serum free triiodothyronine (FT₃), free thyroxin (FT₄), resistin and leptin levels of obesity rats which treated with 50mg rutin/kg b.wt/day dependent on the time of treatment (30 & 60 days). Due to the synergistic effects of ozonized water, the best amelioration results was obtained in the obesity rats group which treated with ozonized water and depending on the time of supplementation (Table 5).
Table (5): Amelioration effect of rutin and ozonized water on hormone profile in obese male rats at various time intervals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Non-obesity normal groups</th>
<th>Obesity groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control rutin</td>
<td>Control ozonized water</td>
</tr>
<tr>
<td></td>
<td>FT3 (pg/ml)</td>
<td>30 days</td>
<td>60 days</td>
</tr>
<tr>
<td></td>
<td>1.25 A ±0.067</td>
<td>1.20 A ±0.069</td>
<td>1.24 A ±0.067</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>1.19 A ±0.061</td>
<td>1.18 A ±0.059</td>
</tr>
<tr>
<td></td>
<td>FT4 (µg/dL)</td>
<td>30 days</td>
<td>60 days</td>
</tr>
<tr>
<td></td>
<td>0.590 A ±0.014</td>
<td>0.602 A ±0.020</td>
<td>0.590 A ±0.019</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>0.582 A ±0.017</td>
<td>0.563 A ±0.015</td>
</tr>
<tr>
<td></td>
<td>Resistin</td>
<td>30 days</td>
<td>60 days</td>
</tr>
<tr>
<td></td>
<td>1.80 A ±0.049</td>
<td>1.78 A ±0.043</td>
<td>1.69 A ±0.046</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>1.85 A ±0.048</td>
<td>1.72 A ±0.051</td>
</tr>
<tr>
<td></td>
<td>Leptin</td>
<td>30 days</td>
<td>60 days</td>
</tr>
<tr>
<td></td>
<td>5.21 A ±0.084</td>
<td>4.90 A ±0.079</td>
<td>4.81 A ±0.089</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>4.62 A ±0.091</td>
<td>4.21 A ±0.065</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE.
- Means with a common superscript within a row are not significantly different (P>0.05).
- Means with a common subscript within a column are not significantly different (P>0.05).

After the obese rats treated with 50mg rutin/kg.b.wt/day for (30& 60) days, the data recorded a significant (P<0.05) increase in the hepatic level of total antioxidant capacity (TAC) and glutathione (GSH) dependent on duration of treatment (Table 6). The maximum elevation was occurred in the hepatic level of TAC and GSH in obese rats group which received ozonized water (Table 6). A remarkable correction was reported in the lipid peroxidation (MDA) after the obesity rats treated with 50mg rutin/kg.b.wt/ day for(30& 60) days (Table
These significant (P<0.05) corrections were pronounced in the obesity rats group which treated with ozonized water.

**Table (6): Amelioration effect of rutin and ozonized water on TAC, GSH and MDA in obese male rats at various time intervals.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Non-obesity normal groups</th>
<th>Obesity groups</th>
<th>Ozonized water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
<td>Control</td>
<td>Recovery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rutin</td>
<td>ozonized water</td>
<td>Obese group</td>
</tr>
<tr>
<td>TAC (mg/g)</td>
<td>30 days</td>
<td>5.69 ±0.21</td>
<td>6.08 ±0.15</td>
<td>5.81 ±0.42</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>6.01 ±0.42</td>
<td>6.26 ±0.50</td>
<td>6.26 ±0.42</td>
</tr>
<tr>
<td>GSH (mg/g)</td>
<td>30 days</td>
<td>14.91 ±1.52</td>
<td>14.61 ±0.01</td>
<td>14.52 ±0.95</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>14.51 ±0.90</td>
<td>14.50 ±0.40</td>
<td>14.31 ±0.92</td>
</tr>
<tr>
<td>MDA (mg/g)</td>
<td>30 days</td>
<td>3.51 ±0.42</td>
<td>3.51 ±0.13</td>
<td>3.61 ±0.12</td>
</tr>
<tr>
<td></td>
<td>60 days</td>
<td>3.56 ±0.30</td>
<td>3.53 ±0.50</td>
<td>3.25 ±0.50</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE
- A, B, C, D Means with a common superscript within a row are not significantly different (P>0.05).
- a, b Means with a common subscript within a column are not significantly different (P>0.05).

**DNA analysis, single-cell gel electrophoresis (Comet assay)**

The results in Table (7) and Figs. (1, 2, 3 and 4) showed that HFD induced a significant concentration dependent increase in the tail length and % of DNA in tail in the liver. Normal rats showed more or less constant levels during the course of the study. Moreover, no remarkable changes were reported after rats were treated with either rutin or ozonized water throughout the experimental duration. Where in the obese rats group which was fed HFD, a significant elevation in the tail length and % of DNA in tail were recorded. The percentage of increment was 222.91% and 495.92% respectively at the end of the experiment (60 days) as compared to their corresponding animals in the normal control. Additionally, the
Supplementation of rutin to obese rats group for 60 days led to a significant decrease in the tail length and % of DNA in tail (Table 7 and figure 3). The percentages of these changes were 85.63% and 166.45% respectively. The maximum correction in the tail length and % of DNA in tail was recorded in the obese rats groups which were treated with ozonized water and this correction depended on the time of supplementation (table 7&figure 4). The percent of correction reached to 51.64% and 63.01% compared to their corresponding value of the normal animals group at the end of the experiment (60 days).

**Table (7): Comet assay parameters by image analysis of cells isolated from liver in control and obesity group.**

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>Non-obesity normal groups</th>
<th>Obesity groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>% of tailed length (damaged cells)</td>
<td>5.50\text{A}_a\pm0.31</td>
<td>5.11\text{A}_a\pm0.3</td>
</tr>
<tr>
<td>% of DNA in tail</td>
<td>3.19\text{A}_a\pm0.68</td>
<td>3.41\text{A}_a\pm0.5</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SE
- A, B, C, D Means with a common superscript within a row are not significantly different (P>0.05).
- a, b Means with a common subscript within a column are not significantly different (P>0.05).
Photograph of fragment DNA migration pattern by comet assay evaluated with a fluorescence microscope for liver cells Fig. (1): control group showing intact cells; most of DNA is located in the head of the comet. Fig. (2): HFD group after 60 days showing tailed cells as a marker of DNA fragmentations; fragmented DNA migrated from the comet head and formed a tail. Fig. (3): obese rats group treated with rutin for 60 days, showing restore the normal intact cells; most of DNA is located in the head of the comet. Fig. (4): obese rat groups treated with ozonized water for 60 days, showing the intact DNA without migration.

**DISCUSSION**

Obesity is the most common nutritional disorder in the world population. It is associated with increased mortality and morbidity of cardiovascular disease (CVD). It is primarily considered to be a disorder of energy balance and it has recently been suggested that some forms of obesity are associated with chronic low-grade inflammation.[30]

Obesity is also a multifactorial disease resulting from a combination and interaction of genetic, environmental, psychological, social and cultural factors.[31] It is a strong risk factor for developing dyslipidemia, diabetes mellitus, fatty liver (which can later progress to nonalcoholic fatty liver disease), cardiovascular (CV) diseases such as heart failure (HF) and coronary heart disease (CHD) and is characterized by increased mass of adipose tissue, which is an active endocrine and secretory organ.[32] Rat models fed with HCD can be used as model of the human obesity syndrome.[33, 34]
In the present investigation, rat was used as an animal model for induction of obesity by feeding HFD. The administration of HFD led to a marked elevation in the body weight due to increased metabolic efficiency of the HFD, the high fat nature of the HFD and also, as a consequence of change in lipid metabolism and storage thus promoting weight gain and installing obesity. These results are in harmony with Lavie et al. and Meziane et al.\[35, 36]\[35, 36\]

The histological study of the liver sections was aimed to support the biochemical investigation. In the present study, histological alteration following HFD feeding in the liver of rats were manifested in the form of inflammatory response with mononuclear cells and lymphocytes and dilatation of sinusoidal space. These changes progressed to generalized hepatic necrosis where hepatocytes were more or less completely vacuolated. In addition cytoplasm of hepatocytes suffered from degeneration. These results were in accordance with those of Hirako et al.\[37\]\[37\] who stated that HFD that causes hepatotoxicity and fatty liver.

Feeding with a fatty diet may cause vascular dilatation in the liver. This dilatation may be caused by inflammatory changes. Similarly, Dai and Chen.\[38\]\[38\] reported expanded sinusoids in the livers of HFD-fed rats. This dilatation may also be caused by ischemia and hypoxia following HFD.\[39, 40\]\[39, 40\] According to different views, vascular dilatation may occur due to developing hypertension after obesity induced by HFD.\[41\]\[41\]

Limited improvement occurred in the histological section of liver after treatment with 50 µg rutin dependent on both time and dose. These results may be due to the unique phenolic properties of rutin which acts as free radicals scavenging. These results were in accordance to those reported by Panchal et al.\[42\]\[42\]

In the present work, it is of interest to ratify that ozonized water treatment showing near to normal hepatic structure after 60\textsuperscript{th} day in obese rats. As far as the present authors are concerned no available data were for ozone therapy concerning histological studies.

It is evident from the present study that there was reciprocal relationship between the concentration of thyroid hormones (FT\textsubscript{3} and FT\textsubscript{4}) in serum and hyperlipidemia depending on the percent of fat content. Present results revealed a significant decrease in the level of FT\textsubscript{3} while only a numerical change occurred in the level of FT\textsubscript{4}. These results seemed to be in complete accordance with earlier studies made by Heibashy et al.\[43\]\[43\] Also, these results may be due to disturbance in hypothalamus-pituitary axis, the conversion of FT\textsubscript{3} to FT\textsubscript{4} or/and
conversion of reverse FT$_3$ to FT$_4$ as a result of feeding rats on HFD causing hypothyroidism. This result confirmed the statement reported by Liu et al.\cite{44} that "FT$_3$ is considered to be the major biologic mediator of the thyroid function test.

On the other hand, the administration of 50µg rutin to obese rats led to improvement in thyroid hormone levels dependent on time and dose. Rutin increases thyroid iodide uptake in rats and decreases serum T$_3$ and T$_4$ level. The decreased hormone level can be explained by its inhibitory effect produced on thyroid peroxidase enzyme (TPO) which is in agreement with Gonçalves et al.\cite{45}

It is clear from the present results that marked recovery occurred in the level of FT$_3$ and FT$_4$ in rats treated with 0.5 ml ozonized water and this recovery dependent on time and dose.

Adipose tissue secretes a number of active peptides and proteins that are collectively known as adipocytokines.\cite{46} Adipocytokines secreted by adipose tissue can function as autocrine substances, paracrine substances or endocrine hormones, and affect metabolic functions of other organs.\cite{47} Among these adipocytokines, there are resistin and leptin. Resistin causes impaired insulin sensitivity and is associated with insulin resistance and obesity. In the present study, HFD feeding is accompanied by an increase in the level of serum resistin due to systematic inflammation that leads to increased resistin production and circulating levels in human. The increased level of resistin in humans with obesity is likely to be an indirect result of elevated levels of inflammatory cytokines characteristic of states of increased adiposity.\cite{48}

In the present study, hyperleptinemia is pronounced as a result of HFD feeding. Prolonged treatment led to a time dependent significant elevation in the level of blood leptin. Plasma leptin concentrations are significantly elevated in obese subjects in proportion to the degree of adiposity, suggesting that hyperleptinemia may play a role in the pathogenesis of obesity-related complications.\cite{49,50}

Under treatment by 50 µg/kg.b.wt rutin for two months treated groups showed dose and time decrease in leptin and resistin levels. These finding may be attributed to inhibition of adipogenesis and adipocytes differentiation. These results come in accordance to those reported by Hsu and Yen.\cite{51}

Considerable improvement occurred in the levels of leptin and resistin in obese rats group treated with ozonized water at dose (0.5 ml / b.wt), this correction depended on the time of
supplementation. Limited literature concerning ozone therapy makes present results as reliable data.

Concurrent results revealed significant elevation in the lipid profile (Cholesterol, triglycerides, LDL-cholesterol and VLDL-cholesterol) and phospholipids levels in HFD rats. These results are in agreement with Woo et al. and Kamal and Mohamed.\textsuperscript{52, 53} They reported that dyslipidemic changes accompany obesity that may be due to the increased triacylglycerol content of the liver due to increased influx of excess non-esterified (free or unsaturated) fatty acids (NEFAs) into the liver;\textsuperscript{54} It has been revealed that altered lipid concentrations and qualitative changes of the lipoprotein fractions in obesity are associated with an increased risk of various adverse effects of obesity.\textsuperscript{55} Additionally, lipid alterations have been considered as contributory factors to oxidative stress in obesity. Increased production of reactive oxygen species as well as reduced antioxidant defense mechanisms have been suggested to play a role in both humans and animal models of obesity.\textsuperscript{56}

Furthermore, obesity and hypercholesterolemia are caused by multiple environmental factors and genetic predispositions. In rodents, hypercholesterolemia occurs due to increased hepatic cholesterol synthesis, decreased LDL-c clearance, conversion of cholesterol to bile acids and secretion of cholesterol into the bile.\textsuperscript{57}

Phospholipids are the major constituents of the biomembrane and are the primary targets of peroxidation process and they are altered by HFD consumption \textsuperscript{58}. In the present investigation, a significant dose and time elevation in hepatic phospholipids with HFD occurred. Administration of HFD increases the biosynthesis of phospholipids possibly by a decrease in phospholipase activity or increased phospholipids turnover due to an onset of inflammatory processes. These results are in harmony with those obtained by Rasmy.\textsuperscript{59}

On the other hand, the administration of rutin at dose 50 mg/kg b.wt. to obese rat groups led to a significant decrease in the levels of cholesterol, triglyceride, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) and hepatic phospholipids but no remarkable change in high density lipoprotein (HDL) level, dependent on time and dose. Several authors recorded that rutin lowers the lipid components in the serum of HFD rats, probably by reducing the activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase \textsuperscript{60}. This may be explained on the basis that rutin has a strong ability to chelate multivalent metal ions, especially zinc, calcium and iron.\textsuperscript{61}
In the current work, the maximum correction was occurred in investigated parameters lipid profile (cholesterol, triglycerides, HDL, LDL and VLDL) and phospholipids in the obese animal groups which treated with 0.5 ml / b.wt ozonized water.

Presently, a significant dose and time decrease of the TAC level take place after HFD feeding dependent on dose and time. Alagumanivasagam et al.\textsuperscript{[62]} reported that HFD can cause the formation of toxic intermediates that can inhibit the activity of antioxidant enzymes and the accumulation of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ which in turn forms hydroxyl radicals.

Under treatment by (50 µg/ kg.b.wt rutin for two months) treated groups showed increase in TAC levels, but the maximum improvement was occurred in rats treated with 0.5 ml ozonized water dependent on time and dose.

Endogenous non-enzymatic defense system against oxidative stress includes the sulphadryl containing peptide namely GSH. It is widely distributed in all biological tissues. GSH inhibits ROS oxidative injuries directly via its sulphhydryl group and indirectly as a cofactor or a coenzyme in ROS enzymatic detoxification process.\textsuperscript{[63]} Furthermore, ROS can harmfully impair lipid contents in cell membrane leading to lipid peroxidation process. Measurements of the endogenous antioxidants as well as lipid peroxidation products, as in case of the current study, have been widely used to assess the degree of cellular oxidative damage. Feeding of experimental animals with HCD was reported to cause oxidative stress and to induce ROS generation in different biological tissues including liver, brain, kidney and erythrocytes.\textsuperscript{[64]} Furthermore, Scheuer et al.\textsuperscript{[65]} showed that HCD altered cellular membrane lipids making the extracellular matrix to be more prone to free radical induced damage. The present study showed similar results, where HFD feeding to rats for six weeks significantly increased lipid peroxidation products (MDA) and decreased the endogenous antioxidant (GSH) in liver tissues.

Rutin is well known for its strong ability to prevent oxidative damage and to scavenge free radicals such as ROS effectively produced through biological processes in many extracellular and intracellular reactions.\textsuperscript{[66]} So, in the concurrent study, treatment with 50 µg rutin increased GSH concentrations in HFD rats.

It is clear from the present results that marked recovery occurred in the hepatic GSH level in rats treated with 0.5ml ozonized water and this recovery dependent on the time of treatment.
The present results revealed significant increase of MDA levels in obese rats. These results are in agreement with Vincent et al and Amirkhizi et al.\(^ {67, 68}\) who showed that obesity is an independent risk factor for increasing lipid peroxidation and decreased activity of cytoprotective enzymes. Obesity can cause increased lipid peroxidation by progressive and cumulative cell injury resulting from pressure of the large body mass. Cell injury causes the release of cytokines, especially tumor necrosis factor alpha (TNF-a) which generates ROS from the tissues which in turn causes lipid peroxidation. The hypertriglyceridemia seen in obese rats may contribute to the alteration in the oxidant-antioxidant balance, suggesting that an increase in the bioavailability of free fatty acids can increase lipid peroxidation.

Recently, Alkhamees.\(^ {69}\) reported that rutin can significantly reduce the elevated hepatic levels of lipid peroxidation biomarker, MDA. This may be because of the naturally occurring phenolic flavonoids, which are characterized by their cytoprotective ability.\(^ {70}\) However, the cytoprotective effects of hydrophobic molecules like rutin were suggested to be through interacting more powerfully with lipids in membranes resulting in more ability to protect cell membranes.\(^ {71}\)

A pronounced recovery was ratified in the level of MDA of obese rat groups which treated with 0.5 ml of ozonized water and this correction depended on the time of supplementation. Leon et al.\(^ {72}\) reported that ozone therapy has been able to preserve liver integrity by inducing either enzymes or activating metabolic pathways that maintain an equilibrated redox balance. High SOD, GSH levels, low peroxidation, catalase and peroxidase are clear examples of the efficacy of ozone therapy.

Owing to its high sensitivity, the Comet assay is considered as a good DNA damage detecting method.\(^ {73}\) The present results of Comet assay show that DNA damage occurred in the liver tissues of HFD rats compared to control rats. Also, as DNA damage is directly proportional to the tail moment, the large value of the tail moments confirms the higher degree of DNA damage in HFD-treated condition.

Free radicals exert their primary effect by reacting with macromolecules including DNA, lipids, proteins and carbohydrates. The oxidative DNA damage plays an important role in the carcinogenic processes. So, oxidation caused by free radicals is one of the major causes of DNA damage in human. Abstraction of hydrogen atoms from unsaturated bonds in lipids is a major reaction of free radicals that result in lipid peroxidation ultimately giving rise to toxic
products such 4-hydroxyalkenals and malondialdehyde.\textsuperscript{[74]} The present results revealed that treatment with 50 \(\mu\)g/kg.b.wt appeared improvement in hepatic damaged cells of obese rats group. This may be attributed to the fact that rutin has displayed protective effects against DNA damage.\textsuperscript{[75]} It is also a protective agent mainly be due to its radical scavenging activity.\textsuperscript{[76]}

Under treatment by (0.5 ml / b.wt. ozonized water for two months) treated groups showing restore the normal intact cells; most of DNA is located in the head of the comet. Due to the paucity of literature concerning ozone therapy only present result were mentioned. Limited available literature for ozone therapy renders present results as reliable data.

In conclusion, it may be substantiated that HFD stimulated different serious effects leading to damage, disturbance in the lipid levels and dysfunction in the liver. Furthermore, rutin treatment for obesity cases has recently proved to be of ultimate benefit. Oxygen therapy on the hand is considered to be a novel method for treatment of different diseases including obesity. Nevertheless, it has not been extensively tackled.

Recently, the supplement of diet with oxygen therapy ameliorates the extent of recovery compared to rutin intake. It plays an important role in minimizing and reducing harmful effects of HFD. Accordingly, present results could be considerable as reliable reference. Where, different parameters have been included to set forth preliminary background for oxygen therapy. Yet, additive studies are required to add to the authenticity of present investigations.

**REFERENCE**


