Original article:
The changes of activity of effector caspase cascade components in case of alimentary obesity in rats

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Abstract:
Objective: The prevalence of obesity is rising in a global fashion, placing this population at higher risk for type 2 diabetes mellitus and cardiovascular disease. Current strategies for treatment of obesity are largely unsuccessful over the long term, since weight loss, even when achieved initially, is often followed by weight regain. More knowledge about the molecular and cellular mechanisms governing adipose tissue accumulation is needed to develop more effective preventative and therapeutic approaches to obesity. Materials and Methods: This study was conducted to investigate the macro- and microelements contents in the blood of experimental animals and assess their influence on the level of apoptotic cell death of blood leukocyte suspension in case of alimentary obesity, which was modelled on male, non-liner, white rats of around 3 months. Annexin V binding assays were performed by flow cytometry, the atomic absorption spectrophotometer was used to quantify micro- and macro-element contents. Results and Discussion: It was established a significant increase in leptin concentration at almost the same level of adiponectin. Experimental obesity was characterized by 2.1 times increasing of Annex+ cells percentage, while the level of PI+ cells remained within the control values. It was established the increasing of total calcium level, which exceeded by approximately 25.0 % of the control data. The same trend was marked on changes of ionized calcium level (p<0.05). So, alimentary obesity in rats is accompanied by the increasing of total and ionized calcium contents and decreasing of iron, magnesium and zinc contents. The combination of the dysmetabolism of investigated bioelements with the maximum effect of zinc affects the apoptotic cells’ death at obesity. Conclusion: Changing of activity of effector caspase cascade components, including caspase - 3 in case of diet-induced alimentary obesity, causes implementing of blood suspension leukocyte cells’ death by apoptosis, and the initiation of apoptosis in this case depends on the body mass index and leptin concentration. The study showed that the combination of the dysmetabolism of investigated bioelements with the maximum effect of zinc affects the apoptotic cells’ death at obesity.

Keywords: obesity, apoptosis, micro- and macro-element contents.
adults succumb to death every year owing to their overweight or obese status. According to WHO, in Ukraine 50.5% of men are overweight, 16% of them are obese and 56% of women are overweight, 26% of them are obese. In general, 45% of people of working age in Ukraine are obese. In the US, more than 50% of the population are overweight, herewith obesity is found in 35% of women and 31% of men. In Europe, the incidence of obesity is 20% in Switzerland, Bulgaria, Italy, France and Spain; 30% in Germany, Finland, the UK and 40% - in Romania. Intercountry comparable overweight and obesity estimates from 2008 show that 58.6% of the adult population (> 20 years old) in Poland were overweight and 25.3% were obese. The prevalence of overweight was higher among men (62.8%) than women (54.7%). The proportion of men and women that were obese was 23.8% and 26.7%, respectively. Adulthood obesity prevalence forecasts (2010–2030) predict that in 2020, 23% of men and 17% of women will be obese. By 2030, the model predicts that 28% of men and 18% of women will be obese.

The proportion of children in the general population who are overweight and obese has doubled over the past two decades in developed and developing countries including India and have a rising prevalence of diabetes. Childhood obesity increases the risk of adult obesity as well as chronic health problems such as type II diabetes, hypertension and cardiovascular disease.

So, the prevalence of obesity is rising in a global fashion, placing this population at higher risk for type 2 diabetes mellitus and cardiovascular disease. Current strategies for treatment of obesity are largely unsuccessful over the long term, since weight loss, even when achieved initially, is often followed by weight regain. More knowledge about the molecular and cellular mechanisms governing adipose tissue accumulation is needed to develop more effective preventative and therapeutic approaches to obesity. At present we know that long-term increased contents of saturated fatty acids in blood activate intracellular ways of cells' damage due oxidative stress. It was shown that increased body mass index in case of alimentary obesity also correlates with mitochondrial dysfunction and increased oxidative stress in the liver. Macro- and microelements play significant role in the free-radical oxidation. Thus, iron and copper, on the one hand, act as a free radical reactions' chains, and zinc and manganese, on another – antioxidants that control oxidative processes. Moreover, it is known that trace elements play crucial role in the process of programmed cell death, for example zinc and magnesium deficiency potentiate apoptosis. Calcium also has important role in the mechanisms of cell death.

We conducted this study to investigate the macro- and microelements contents in the blood of experimental animals and assess their influence on the level of apoptotic cell death of blood leukocyte suspension in case of alimentary obesity.

Materials and methods.

Experimental studies were conducted on male, non-liner, white rats of around 3 months of age, that were housed at 25±3 °C and humidity of 55±2 %, under a constant 12 h light and dark cycle. Water was available ad libitum. Experimental obesity was modelled by including sodium glutamate to the feed mixture in a ratio of 0.6:100.0 and using a high-calorie diet that consists of the standard meal (47%), sweetened concentrated milk (44%), corn oil (8%) and vegetable starch (1%) (diet # C 11024, Research Diets, New Brunswick, NJ, USA). When selecting for the optimal model of alimentary obesity, we stressed on the fact that sodium glutamate affects ventrolateral nuclei of the hypothalamus, where the hunger centre is located, and thus it stimulates appetite. To monitor the alimentary obesity model, animals were weighted, their nasal-anal length measured, and body mass index (body weight in kg divided by the squared length in meters) calculated. The experimental animals were divided into the following groups: in group 1, the rats that were on the experimental diet for 14 days (EG 1), and in group 2, for 28 days (EG 2). The control groups (CG) consisted of animals maintained on a standard diet for 14 days (CG 1) and 28 days (CG 2), respectively. Animal euthanasia was carried out on the 14th and 28th days of the experiment by cardiac puncture under deep anaesthesia, in accordance with the requirements of the Animal Care Committee. Blood was collected in two different tubes (i.e.,) one with anticoagulant- potassium oxalate and sodium fluoride for plasma and another without anticoagulant for serum separation. Plasma and serum were separated by centrifugation.

Blood glucose was determined by the method of O-toluidine using the modified reagent. Plasma insulin, adiponectin and leptin levels were assayed by Enzyme Linked Immunosorbent Assay (ELISA) kit. The serum concentrations of triglycerides and the total cholesterol were estimated using reagents from Biochemical diagnostic kits BIO-LA-TEST (Erba Group). Serum total iron concentration, total and ionized calcium concentration were measured by...
semi-automated biochemical analyzer Humalyzer 2000 (Human, Germany).
Annexin V binding assays were performed using Annexin V Apoptosis Kit (Sigma Aldrich, USA). Apoptotic cells were identified by flow cytometry. To distinguish cells that had lost membrane integrity, propidium iodide (PI) was added to a final concentration of 10 mg/mL before analysis. To determine caspase rate in leukocyte-lymphocyte blood fractions, 0.25 ml of buffer and 50 mcl of 2 mM DEVD-p-NA was added to 0.7 ml of the test liquid. It was incubated for 2 hours at 37°C; the intensity of light absorbance was measured at 405 Nm, which is directly proportional to the product of hydrolysis of Acetyl-Asp-Glu-Val-Asp n-nitroanilide caspase – 3-n-nitroanilide.

The atomic absorption spectrophotometer with flame and graphite furnace was used to quantify micro- and macro-element content. For simultaneous analysis, it consists of eight turret lamps with a wavelength range of 190–900 nm. The spectroscopic conditions were the following: bandwidth 0.4 nm with a 1.0 filter factor for zinc and magnesium, bandwidth 0.1 nm with a 1.0 filter factor for calcium and copper. The integration time was 3.0 s set at 5.0 mA lamp current for calcium and zinc, at 3.0 mA lamp current for magnesium and 2.0 mA lamp current for copper. The elements were detected at the following wavelength: calcium – 422.7 nm, magnesium – 285.2 nm, zinc – 213.9 nm, and copper – 324.7 nm.

Statistical analysis. The results were analysed using Statistica 6.1 software and presented as mean with standard error of mean. The differences between all groups were determined using one-way ANOVA, followed by post hoc Least Significant Difference test, with p<0.05 considered statistically significant.

**Results.**

It was found that the weight of the rats with alimentary obesity after 28 days of observation almost twice exceeded the rate in animals on standard diet (p<0.05). Data analysis of lipid and carbohydrate metabolism showed significant increase in total cholesterol level by 75.5 % and triglycerides level by 74.7 % vs control group (p<0.01) on the background of normal levels of glucose, insulin and insulin resistance index in the modeled pathology. Adiponectin and leptin, which are produced by adipose tissue, also play an important role in the metabolism of proteins and carbohydrates. It was established a significant increase in leptin concentration (in 2.5 times) at almost the same level of adiponectin. It was accompanied by a decrease in the Adiponectin / leptin index (in 2.7 times) vs control group (p<0.01) (table 1).

**Table 1: The Indices of Lipid and Carbohydrate Metabolism in Blood of Rats with Diet-Induced Obesity**

<table>
<thead>
<tr>
<th>Index</th>
<th>Control group (n=6)</th>
<th>Experimental group 2 (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>7.95±0.45</td>
<td>8.45±0.32</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.10±0.37</td>
<td>8.95±0.44*</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.70±0.30</td>
<td>2.97±0.45*</td>
</tr>
<tr>
<td>Insulin, µU/ml</td>
<td>3.95±0.45</td>
<td>4.29±0.36</td>
</tr>
<tr>
<td>HOMA – IR</td>
<td>1.42±0.22</td>
<td>1.59±0.11</td>
</tr>
<tr>
<td>Leptin, pg/ml</td>
<td>126.97±8.49</td>
<td>315.48±11.74*</td>
</tr>
<tr>
<td>Adiponectin,ng/ml</td>
<td>191.67±6.29</td>
<td>177.58±13.24</td>
</tr>
<tr>
<td>Adiponectin / Leptin</td>
<td>1.53±0.06</td>
<td>0.57±0.04*</td>
</tr>
</tbody>
</table>

Note: * – significant difference comparing with the control group (p<0.05).

To investigate the mechanism of apoptosis’ onset, the percentage of PI+ and Annex+ blood leukocytes cells’ suspension at alimentary obesity was determined. It was established the increasing of Annex+ cells percentage by 1.6 times within 14 days of the experiment, while PI+ cells percentage remained within the control values.

Alimentary obesity modeled after 28 days of hypercaloric diet was characterized by 2.1 times increasing of Annex+ cells percentage, while the level of PI+ cells remained within the control values. It should be noted that the percentage of apoptotic cells after 28 days was 58.4 % higher than the findings after 14 days (p<0.05) (figure). A direct correlation of apoptosis with concentrations of leptin and BMI was established.

Caspases in general are important mediators in apoptosis, especially caspase-3. It was established the increasing activity of effector caspase-3 after 28 days of experiment by 2.2 times vs control group, which indicates on caspase-dependent apoptotic pathway in case of alimentary obesity in rats (figure).
The changes of activity of effector caspase cascade components in case of alimentary obesity in rats

Figure. The indices of death of blood leukocytes cells’ suspension in case of alimentary obesity in rats (*-- significant difference comparing with the control group).

The next stage of our study was to determine the content of some macro- and microelements in the blood of rats with diet-induced obesity (table 2). It was established the increasing of total calcium level in 2-nd experimental group, which exceeded by approximately 25.0 % of the control data and data of the first experimental group. The same trend was marked on changes of ionized calcium level, which after 28 days of the experiment increases approximately by 56.0 % vs control group and data after 14 days (p<0.05). The analysis of magnesium level showed its decreasing in second experimental group by 36.0 % vs control group and the first group (p<0.05). In case of the diet-induced obesity it was established the decrease of serum iron by 9.4 % in the second experimental group vs control group. Reduction of zinc in animals with modeled pathological process was observed within 14 days of the experiment (by 21.2 %) with a continuation of its decreasing to the 28th day (by 38.0 %).

Table 2: Indices of Some Macro- and Microelements in Blood of Rats with Diet-Induced Obesity

<table>
<thead>
<tr>
<th>Index</th>
<th>Control group (n=6)</th>
<th>Experimental group 1 (n=6)</th>
<th>Experimental group 2 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total calcium, mmol/l</td>
<td>2.30±0.04</td>
<td>2.33±0.03</td>
<td>2.87±0.05</td>
</tr>
<tr>
<td>p1&gt;0.05</td>
<td></td>
<td>p1&lt;0.05; p2&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Ionized calcium, mmol/l</td>
<td>0.64±0.02</td>
<td>0.68±0.01</td>
<td>1.00±0.03</td>
</tr>
<tr>
<td>p1&gt;0.05</td>
<td></td>
<td>p1&lt;0.05; p2&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Iron, mmol/l</td>
<td>8.79±0.11</td>
<td>8.59±0.11</td>
<td>7.96±0.10</td>
</tr>
<tr>
<td>p1&gt;0.05</td>
<td></td>
<td>p1&lt;0.05; p2&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Magnesium, mg/g</td>
<td>0.72±0.02</td>
<td>0.75±0.04</td>
<td>0.46±0.03</td>
</tr>
<tr>
<td>p1&gt;0.05</td>
<td></td>
<td>p1&lt;0.05; p2&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Zinc, µg/g</td>
<td>1.37±0.05</td>
<td>1.08±0.03</td>
<td>0.85±0.03</td>
</tr>
<tr>
<td>p1&lt;0.05</td>
<td></td>
<td>p1&lt;0.05; p2&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Notes: 1. p1 – significant difference comparing with the control group; 2. p2 – significant difference comparing with affected animals.

Discussion.

A significant increase in leptin concentration and a decrease in the adiponectin / leptin index in our investigation coincide with the results of other studies. Thus, in case of the experimental diet-induced obesity, high level of leptin is not able to function, in particular, to induce weight loss through leptin resistance. The increase in carbohydrate metabolism indices, in our opinion, is associated with the reduction of the central action of leptin. Results of the Unger R.H. study indicate that the decreasing of the central action of leptin causes deterioration of insulin secretion and sensitivity, which is consistent with our data.

Low level of adiponectin, which was found, according to some authors, is a prerequisite for insulin resistance and type 2 diabetes. Recent studies indicate the different effects of leptin on apoptosis. So, has been shown ability of leptin to exhibit antiapoptotic activity in T cells, monocytes, and neuroblastoma cells, and on the other hand, has been reported ability of leptin to induce apoptosis in human bone marrow stromal cells. Study of Gullicksen P.S. et al. indicates that leptin acts centrally to trigger an apoptotic process resulting in adipocyte deletion. A direct correlation of apoptosis with concentrations of leptin and BMI was established. These data suggest that leptin triggers apoptotic cell death, as noted by some authors, still through unknown mechanisms.

Two known ways of apoptosis include internal or mitochondrial, involving protein family Bcl–2, cytochrome C and caspase – 9 and external with the activation of caspase – 8 linking a specific cell receptor Fas– and soluble tumor necrosis factor receptors on the cell surface. Caspase-3 is the most involved pathway which should be generated from its inactive protein (procaspase-3), caspase 3 is required for some apoptosis features (chromatin condensation, DNA damage and apoptotic body formation) and its role may take place before cell viability suppression starts.

Our research showed the increase of total and ionized calcium in the blood serum of experimental animals. It can be explained by osteoclastic activity and bone...
resorption due to increasing of proinflammatory cytokines’ contents in case of alimentary obesity. Several authors have established the increasing of calcium level in the blood together with the increasing levels of triglycerides, although there is no mechanism of interdependence. The analysis of magnesium level showed its decreasing in second experimental group. One of the mechanisms of such changes in different directions of these two microelements in case of alimentary obesity is the development of inflammation, as evidenced by the increasing of tumor necrosis factor-alpha. Overbergh L. et al. in their study showed that tumor necrosis factor activates renal 1-α-hydroxylase and 1.25 (OH)2 vitamin D, which increase the reabsorption of calcium in the kidneys and excretion of magnesium. The decrease of serum iron in the second experimental group can be explained by the increasing of hepsydis contents - regulatory protein that is produced not only by hepatocytes, but by adipose tissue, and directly reduces iron absorption in the small intestine and blocks its excretion out of the macrophages. Our assumption is confirmed by the results of several studies on hepsydis-mediated inhibition of iron absorption from food in obese patients. Reduction of zinc in animals with modeled pathological process can be attributed to the fact that zinc is likely mediator for leptin effects. The analysis of changes in macro- and microelements in the blood of rats showed that the greatest scope of changes is typical for magnesium (40.0 %), while the lowest - for serum iron (7.1 %). This fact gave reason to include in the equation of multiple regression ANOVA value of apoptosis as effective sign for creation of model, and include contents of calcium, magnesium and zinc as general factors. The data indicate that in case of experimental alimentary obesity on the initiating of apoptotic cell death affect all bioelements, which were investigated, with the maximum effect of zinc. The results were comparable with published data which indicate that zinc may act as a survival factor, and therefore this trace element deficiency hypothesis according to the programmed cell death, can cause apoptosis and necrosis.

**Conclusion:**
Changing of activity of effector caspase cascade components, including caspase - 3 in case of diet-induced alimentary obesity, causes implementing of blood suspension leukocyte cells’ death by apoptosis, and the initiation of apoptosis in this case depends on the body mass index and leptin concentration. Alimentary obesity in rats is accompanied by the increasing of total and ionized calcium contents and decreasing of iron, magnesium and zinc contents. The study showed that the combination of the dysmetabolism of investigated bioelements with the maximum effect of zinc affects the apoptotic cells’ death at obesity.
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References:


