SPECIFICITY OF METABOLIC SYNDROME MODEL REPRODUCTION AT PUBERTAL AND ADULT MALE RATS


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Abstract

Background and Aims: Comparative estimation of metabolic syndrome (MS) mediated changes of blood, cardio-vascular system, liver, pancreas and kidneys morphologic structure in adult and pubertal rats. Materials and Methods: Wistar albino male rats of two age categories (young animals of 21 days age (50-70g) and adults (160-180g)) were divided into 4 groups (8 animals in each): 1 – Control 1 (intact young rats); 2 – Control 2 (intact adult rats); 3 – MS3 (young rats with MS) and 4 – MS4 (adult rats with MS). The metabolic syndrome model was induced by full replacement of drinking water with 20% fructose solution (200g/l). After 60 days of MS modeling, determination of rat hematological and serum biochemical parameters, glucose tolerance, blood pressure, liver rates of lipid peroxydation and chromatin DNA fragmentation, as well as morphological macroscopic and microscopic studies were carried out. Results: In pubertal rats, glucose tolerance, hypertension, blood clotting disturbances, DNA-fragmentation and lipid peroxydation rates were affected more profoundly, while mature rats showed greater Pseudo Pelger-Huet anomaly development, serum cholesterol and lipoproteins increases, liver and kidney morphology changes. Conclusions: Our current data combined with previous results of other authors allow us to conclude that an animal model (Wistar rats) of MS is quite easily obtained in a full age range, from juvenile to mature rats.

key words: metabolic syndrome, pubertal, adult, rats

Background and Aims

Metabolic syndrome (MS) combines several metabolic alterations including glucose intolerance, insulin resistance, hyperinsulinemia, central adiposity, dyslipidemia, arterial hypertension, atherosclerosis, proinflammatory status, microalbuminuria, and obesity [1]. Its pathogenesis is not clearly understood and very complex. However, present data indicate the existence of some age-related features in MS development [2]. These investigations indicated the availability of Wistar rats as an animal model for MS analysis during aging.

In humans, during the previous decades, this pathology affected mainly adults. However, lifestyle changes during the last decade determined MS rapid spread among children and
teenagers [3]. This is a growing public health problem all over the world. Information on MS particularities in children and teenagers is quite limited, whereas its consequences are extremely serious. Such shift towards younger ages of MS raised our special interest in the possibility of using juvenile and pubertal Wistar rats as an animal model for MS. The aim of our study was to carry out a comparative estimation of MS mediated changes of blood, cardio-vascular system, liver, pancreas and kidneys morphologic structure in adult and pubertal rats.

**Materials and Methods**

A total of 32 Wistar albino male rats of two age categories (young animals of 21 days age (50-70g) and adults (160-180g)) were used in the study. They were kept under a controlled temperature (from 22°C to 24°C), relative humidity of 40 % to 70 %, lighting (12 h light-dark cycle), and on a standard pellet feed diet (“Phoenix” Ltd., Ukraine). The study was performed in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and approved by the Institutional Animal Care and Use Committee. The model of metabolic syndrome (MS) was reproduced according to the protocol of Abdulla et al. [4]. Young and adult animals were divided into 4 groups (8 animals in each group): 1 – Control 1 (intact young rats), 2 – Control 2 (intact adults), 3 – MS3 (young rats with MS) and 4 – MS4 (adult rats with MS). Metabolic syndrome was induced by full replacing of drinking water with 20% fructose solution (200g/l). Crystalline D-fructose >99% (Khimlaborreactiv, Ukraine, series 072000897834, batch XW 130105) was used in experiments. 20% fructose (instead of drinking water) was prepared daily and was given every day for two month ad libitum.

**Glucose tolerance determination** was carried out according to Guskova’s method [5]. Animals were fasted 24 hours before the experiment. Glucose load was performed as single oral administration of 40% glucose solution in dose 5 g/kg of body weight. Blood samples were collected from tail vein before glucose load and than after 60, 120, 180 and 240 min. Blood glucose levels monitoring during tolerance test was carried out with Smartest Optima (Biotest T Medical Corporation, Germany).

Changes of rats’ blood pressure were investigated as described by Khromov et al. [6]. Noninvasive investigation of all animals’ blood pressure (96-72 hours before euthanasia) was carried out on tail artery with ultrasound sensor of vascular pulsation using a SPHYGMOMANOMETER S-2 (HSE, Germany). Vascular pulsation monitoring was carried out by oscilloscope HM 303-4 (HAMEG GmbH, Germany). Data were analysed using “Chart 5” (ADInstruments, Australia).

Determination of rat hematological and clinical biochemistry parameters, glucose tolerance, blood pressure, rat liver rates of lipid peroxydation (LPO) and chromatin DNA fragmentation (as one of apoptosis markers), as well as morphological macroscopic and microscopic studies were carried out after 60 days of MS modeling. Both the experimental and control rats were sacrificed under a mild ether anesthesia by decapitation. The blood, serum and organs were used for investigation. Rat liver microsomes were isolated according to procedure described by Kamath et al. [7]. Blood samples were studied with the hematology analyzer Mythic 22, Switzerland, blood clotting time – by standard clinical Burker’s method. Peripheral blood smears were stained by modified Giemza’s method [8]. Serum levels of total bilirubine, total cholesterol, LDL and HDL cholesterol were measured with a fully automatic
biochemical analyzer Prestige 24i (Japan) using kits supplied by “P. Z. Cormay”, Poland. Abdominal fat, liver, kidneys and pancreas of all animals were extracted, weighted and used for morphologic investigation. Relative organ weights were calculated per 100 g of body weight. Pieces of extracted organs were fixed in 10% solution of neutral formalin, dehydrated in ethanol solutions and embedded in paraffin wax. Histological cross-sections (6μm) were made and stained by hematoxilin and eosine. Histological examination was performed under a light microscope (100 x, 200 x and 400 x). In frozen (-20°C) slices of organs, neutral fat was determined by Sudan black B staining [9]. Nucleic acids content (DNA and RNA) was determined histochemically by Shubich method [9], glycogen – by PAS-reaction [10], succinate dehydrogenase activity – by the method of Nachlas et al. [10] and lactate dehydrogenase activity - by the method of Hess et al. [9]. Microscopic studies were carried out with a Cytophan microscope (Leica Microsystems Wetzlar GmbH). DNA from rat liver was isolated as previously described [11]. The levels of lipid peroxidation (LPO) in liver microsomes were investigated as the rates of NADPH-dependent thiobarbituric acid reactive substances (TBARS) formation [12].

Statistical analysis. The obtained data were expressed as the mean ± standard error of the mean (M±SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s test using OriginPro 7.5 Software. Differences were considered to be statistically significant at p < 0.05.

Results

Glucose tolerance (Figure 1) was detected in vivo in rats of both age ranges. Glucose intolerance developed more rapidly in pubertal animals in comparison with adults: at 3-rd and 4-th hours from the beginning of the experiment respectively.

Results of rat blood pressure changes are presented in Figure 2. These data demonstrate that in rats with MS hypertension developed regardless of age.

Hematological investigation showed that platelet count, white blood cell count and red blood cell count in rats with MS did not change significantly in comparison with both controls.
(adult and pubertal), while hematocrit lowered from 41.03±0.78% in controls to 38.45±0.63% in MS (p<0.05) in adult rats and from 43.12±0.60% in control to 39.82±0.94% (p<0.05) in pubertal rats with MS. With MS development, we registered great changes of blood clotting time both in adults and pubertal animals. In adult MS rats blood clotting time was lowered 1.7 times, while in pubertal MS rats it was lowered 2.2 times in comparison with controls.

Microscopic investigation of blood smears allowed us to evidence the development of PPHA in MS rats. Pseudo Pelger-Huet anomaly (PPHA) neutrophils were identified by their uni-lobed or symmetric bi-lobed nuclei, abnormally clumped chromatin, and relatively abundant cytoplasm with pink or yellowish granules. 200 neutrophils in peripheral blood smears were examined, and proportions of PPHA cells were calculated as percentage of total neutrophils. In adult rats PPHA cells accounted for 25.08±0.85 % of total neutrophil count while in pubertal rats for 3.67±0.45% of total neutrophil count (Figure 3). In adult controls such changes of cells chromatin accounted 0.25±0.11 % of total neutrophil count while in pubertal rats they were absent.

![Figure 2. Adult and pubertal animals blood pressure (mmHg) levels, M±SEM, n=8, * - p<0.05 in comparison with control.](image)

![Figure 3. PPHA in adult (a) and pubertal (b) rats with MS.](image)
Table 1. Clinical biochemistry parameters of adult and pubertal animals in norm and with MS, (M ± SEM; n = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Adults</th>
<th>MS Control</th>
<th>Pubertal Adults</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bilirubine, µmol/L</td>
<td>0.73±0.19</td>
<td>1.82±0.16*</td>
<td>0.47±0.17</td>
<td>0.76±0.25</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>1.21±0.08</td>
<td>1.66±0.09*</td>
<td>0.98±0.10</td>
<td>1.38±0.14</td>
</tr>
<tr>
<td>HDLc, mmoles/L</td>
<td>0.92±0.05</td>
<td>1.13±0.09</td>
<td>0.87±0.04</td>
<td>1.14±0.007*</td>
</tr>
<tr>
<td>LDLc, mmoles/L</td>
<td>0.16±0.06</td>
<td>0.36±0.04*</td>
<td>0.16±0.003</td>
<td>0.25±0.006*</td>
</tr>
<tr>
<td>LDLc/HDLc ratio</td>
<td>0.16±0.05</td>
<td>0.33±0.04*</td>
<td>0.19±0.009</td>
<td>0.23±0.016*</td>
</tr>
</tbody>
</table>

* - p<0.05 in comparison with control

Data of rats’ serum biochemical parameters investigation are presented in Table 1. Pubertal animals with MS had higher levels of HDLc and LDLc in comparison with controls. Adult rats with this pathology had two times higher levels of LDLc and also higher levels of total bilirubin and total cholesterol.

Morphological studies demonstrated that in both pubertal and adult MS rats, hepatocytes had focuses of necrobiotic changes and neutral fat accumulation (Figure 4). However, in pubertal MS rats these changes were less profound.

At the same time, in the pancreas of pubertal and adult rats, the number of β–cells in the islets of Langerhans was decreased as well as the number of their secretory granules therein (Figure 5). Simultaneously the number of single β-cells, intensely stained by aldehyde-fuchsine into violet-blue color, was increased. Such changes were observed with the same frequency in both age groups.

Figure 4. Liver of rats with MS.

a – adult rat necrobiotically changed hepatocytes (hematoxilin-eosine staining, x200); b – adult rat hepatocytes neutral fat accumulation (Sudan black B, x200); c – pubertal rat necrobiotically changed hepatocytes (R- stellar reticular endotheliocytes activation, M-increased number of mononuclears, hematoxilin-eosine staining, x200); d – pubertal rat hepatocytes neutral fat accumulation (Sudan black B, x200).
Nephron segments and collecting tubules of both of pubertal and adult rats with MS showed no significant destructive changes. But it should be noted that some animals of both ages had such minor degenerative changes as patchy congestion of glomerular capillaries (more profound in adults), partial detachment of nephrocytes distal parts in proximal tubules, with nucleuses localization at basal membranes. Tubular lumens contained reticulate-granular masses as shown in Figure 6.

Kidneys of control animals (both adult and pubertal) contained neutral mucopolysaccharides in glomerules, proximal segments of convoluted and straight tubules, collecting tubules basal membranes (according to PAS reaction test). The most profound PAS-positive reaction was detected in the apical parts of proximal convoluted tubules nephrocytes (Figure 6a). Experimental animals kidneys (both adult and pubertal) contained reduced quantities of neutral mucopolysaccharides in some proximal tubules (even till their complete absence). Some nephrocytes tubules apical parts had a scalloped structure (Figure 6b).

The relative organs weight and abdominal fat weight of MS adult and pubertal animals were also modified compared to control rats as shown in Table 2.
Table 2. Relative organ weights and abdominal fat weights of adult and pubertal rats with MS and controls.

<table>
<thead>
<tr>
<th>Organs</th>
<th>Adult animals</th>
<th>Pubertal animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MS</td>
</tr>
<tr>
<td>Liver</td>
<td>3.3±0.18</td>
<td>3.18±0.10</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.2±0.01</td>
<td>0.17±0.009*</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.61±0.022</td>
<td>0.63±0.015</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>3.58±0.25</td>
<td>4.81±0.40*</td>
</tr>
</tbody>
</table>

* - p<0.05 in comparison with control

Liver cells structure violations caused by MS were accompanied by intensification of nuclear DNA fragmentation. Liver DNA fragmentation of adult and pubertal rats with experimentally induced MS was significantly higher than in controls as shown in Figure 7. In the group of adult rats with MS we registered 9 fractions of low molecular weight DNA fragments, while in the control group – only two. MS mediated changes in the group of pubertal rats were even more pronounced. We registered 13 fractions of low molecular weight DNA fragments compared to only 1 in controls. A majority of animals in both groups had those fractions that contain low molecular weight fragments (from 20 to 250 base pairs).

Figure 7. Liver DNA fragmentation in normal and MS rats.

Simultaneously, during the MS development, LPO processes in adult and pubertal rats liver cells were intensified (Figure 8) as indicated by the increased rates of NADPH-dependent thiobarbituric acid reactive substances production. In adult rats such increase reached 59% while in pubertal rats 118%.

Figure 8. NADPH-dependent LPO in rat liver microsomal fraction with MS (M ± SEM; n =6).

Discussion

Our data on glucose tolerance, hypertension, serum biochemical parameters and morphological changes are the clear evidence of this MS model correctness in both age group rats. They are in good accordance with other authors results [1-4, 13].

As in a previous study in adult and mature animals [2], Wistar rats were a good model for MS. But it should be stressed that the glucose tolerance, hypertension and blood clotting changes were more pronounced in the younger age rats. This is obviously related not only with general higher rates of metabolic processes in pubertal organisms (both animals and humans) but also with higher basal rates of
glucose conversion to neutral glyceride in them and their higher responsiveness to exogenous insulin [14,15]. On the other hand, biochemical changes in adult rats serum affected not only different fractions of lipoproteins and their ratio, but also bilirubin.

For normal growth and development of young organisms, the maintenance of muscular and adipose tissues in a certain ratio is of fundamental importance. Apart fatty acids, adipose tissue can secrete some energy metabolism regulators - adipokines and hormones [16]. Our morphologic studies demonstrated increasing of pubertal rats abdominal fat mass and liver relative weight (in comparison with controls), while adult rats had only increased abdominal fat mass. Pubertal rats livers microscopy studies have detected (less manifested than in adult rats) hepatocytes dystrophic changes, acidophilic necrosis, fatty drops in hepatocytes cytoplasms (as marker of steatosis development), activation of stellar reticular endotheliocytes (as fibrosis marker), as well as changes of glycogen contents. Pancreas MS associated negative changes were equally expressed in both age groups. In kidneys, damage of nephrons was more profound in adult rats with MS.

Rats of both age groups exhibited a decrease in hematocrit (%), which could be an indication of organisms’ hemorheological profile violations. Among these, the presence of PPHA in blood of adult and pubertal rats with MS indicates possible serious MS mediated changes of nuclear chromatin structure and functions [17], previously registered also in diabetes [18]. Our data are in good accordance with other authors’ results on hemotological indices changes in humans with MS [19,20].

Alterations of any intracellular macromolecules (DNA, RNA, proteins, lipids) structures mediated by MS could play a key role, causing cell damage and death. There is a clear link between DNA structure systemic disorders and a number of indices characterizing the MS development [21]. In MS, liver DNA fragmentation levels were greatly increased in rats of both age groups. As the rate and character of DNA fragmentation is one of the cells apoptosis development markers [22], such data could indicate stimulation of apoptotic processes in pubertal and adult rats with MS. This assumption is confirmed by results of PPHA in blood smears of pubertal and adult rats with MS, as such violations of chromatin structure are associated with stimulation of apoptotic processes [17]. Obtained data are also in good accordance with other author’s results on the strong relationship between MS development and the level of NEIL1 endonuclease activity. This is involved into mammalian cells DNA repair processes [23], and inhibition of nuclear DNA structure repair by nuclear DNA-polymerases, accompanied by mitochondrial dysfunction, neurodegenerative processes and arterial wall pathologies [24,25]. In our study, the relative (%) DNA fragmentation and number of fragments in group of pubertal rats with MS were higher than in adults.

The mechanisms of the observed alterations caused by MS remain insufficiently studied and need further in-depth investigation. At present, LPO is regarded as one of the most probable ones. In our experiments, we have registered increased rates of TBARS-reagents production in both groups, but changes in young animals were 2 times higher than in adults. Probability of LPO products participation in MS pathogenesis is confirmed not only by our data, but also by results of other authors, which have demonstrated MS related stimulation of oxidative stress with simultaneous damages of endothelial cells in adult rats [26]. Another possible mechanism of MS mediated changes is
associated with oxidative stress [27]. Thus, abnormalities in cells superoxide anion production are accompanied by morphological anomalies of the neutrophils such as a PPHA. In turn, this pathology is closely related to changes in levels of lipid and cholesterol production [28]. Cholesterol biosynthesis impairments disrupt neutrophil nuclear lobulation characteristic of PPHA via influence on inner nuclear membrane protein - the lamin B receptor (LBR), which combines an ability to interact with chromatin and lamins, and an enzymatic function as a sterol reductase.

Thus, in our experiments, MS mediated disorders in the metabolism of lipoproteins and cholesterol associated with stimulated LPO possibly caused subsequent deviations in nuclear chromatin structure and functions. And this in turn led to PPHA development in rats with MS. Such supposition also explains more expressed PPHA at older age, when changes of lipid and cholesterol metabolisms were stronger. Thus, the data above suggest the existence of certain age-related effects in the development of MS markers in Wistar rats.

Conclusions
In experiments with MS rats of different age categories we revealed that MS effects in pubertal and adult animals differed significantly. In pubertal rats, glucose tolerance, hypertension, blood clotting, DNA-fragmentation, LPO rates were affected more profoundly, while mature rats showed greater PPHA development, serum total and LDL cholesterol increases, liver and kidneys morphology changes.

In summary, our current data combined with previous results of other authors allow us to conclude that an animal model (Wistar rats) of MS is quite easily obtained in a full age range, from juvenile to mature rats.

REFERENCES
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