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Features of the Proinflammatory Adipose Tissue Phenotype in Experimental Metabolic Syndrome

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ABSTRACT

Aim. To investigate the proinflammatory activity markers of adipose tissue in a rat model of metabolic syndrome (MetS).

Materials and methods. The experiments were performed on male Wistar rats fed with either a standard diet or a high-fat, high-carbohydrate diet (HFHCD). Physiological and biochemical parameters were assessed in the respective animal groups. The concentrations of adipokines (leptin and adiponectin) and cytokines: tumor necrosis factor alpha (TNF α), interleukins (IL-6 and IL-10) and monocyte chemoattractant protein-1 (MCP-1) secreted by adipose tissue cells were determined using enzyme-linked immunosorbent assay. The phenotypic profile of adipose tissue cells expressing the CD68 marker was examined by immunohistochemistry, and the level of *CD68* gene expression was additionally assessed using real-time polymerase chain reaction. A histological examination of visceral adipose tissue fragments was performed.

Results. It was found that in rats fed with a HFHCD, there was a significant correlation ($p < 0.05$) between an increase in adipose tissue mass and elevated serum levels of leptin ($r = 0.57$), glucose ($r = 0.62$), and insulin ($r = 0.61$). In the experimental group, the concentrations of proinflammatory cytokines (IL-6, TNF α , and MCP-1), and leptin produced by adipose tissue cells were higher than in the control group ($p < 0.05$). Additionally, we demonstrated that rats fed with a HFHCD exhibited a significant ($p < 0.05$) decrease in the secretion of the anti-inflammatory cytokine IL-10 and adiponectin. Histological examination of the experimental group revealed adipocyte hypertrophy and anisocytosis, venous vessel congestion, and thickening of the interlobular connective tissue layer. Furthermore, rats receiving a HFHCD exhibited an increased number of CD68-positive cells in visceral fat samples and elevated *CD68* gene expression.

Conclusion. Thus, in experimental diet-induced metabolic syndrome, adipose tissue acquires a proinflammatory phenotype due to adipocyte hypertrophy, infiltration by immune-competent cells (CD68+ macrophages), and increased secretion of proinflammatory chemo- and adipokines (TNF α , IL-6, MCP-1, and leptin).

Keywords: metabolic syndrome, obesity, inflammation, cytokines, macrophages

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Особенности провоспалительного фенотипа жировой ткани при экспериментальном метаболическом синдроме

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РЕЗЮМЕ

Цель. Изучение показателей провоспалительной активности жировой ткани в экспериментальной модели метаболического синдрома (МС) у крыс.

Материалы и методы. Эксперименты выполняли на самцах крыс линии Вистар, которых содержали на стандартном корме или высокожировой и высокоуглеводной диете (ВЖВУД). У животных из опытной и контрольной групп оценивали физиологические и биохимические показатели; определяли концентрацию секретируемых клетками жировой ткани адипоцитарных гормонов (лептина, адипонектина) и цитокинов: фактора некроза опухоли альфа (TNF- α), интерлейкинов (IL) (IL-6, IL-10), моноцитарного хемотаксического фактора-1 (MCP-1) методом иммуноферментного анализа; исследовали фенотипический профиль клеток жировой ткани по экспрессии маркера CD68 методом иммуногистохимии и уровень экспрессии гена *CD68* методом полимеразной цепной реакции. Для анализа и оценки выраженности морфологических изменений проводили гистологическое исследование фрагментов висцеральной жировой ткани.

Результаты. Установлено, что у крыс, получавших ВЖВУД, наблюдалась статистически значимая ($p < 0,05$) корреляция между увеличением массы жировой ткани и повышением концентрации лептина ($r = 0,57$), глюкозы ($r = 0,62$), инсулина ($r = 0,61$) в сыворотке крови. У животных опытной группы концентрация продуцируемых клетками жировой ткани провоспалительных цитокинов IL-6, TNF- α , MCP-1 и лептина превышала таковую в контроле ($p < 0,05$). Наряду с этим у крыс на фоне ВЖВУД было зарегистрировано статистически значимое ($p < 0,05$) снижение секреции противовоспалительного IL-10 и адипонектина. При гистологическом исследовании у животных опытной группы были выявлены гипертрофия и анизоцитоз адипоцитов, полнокровие венозных сосудов, утолщение прослойки междольковой соединительной ткани. Также у крыс, получавших ВЖВУД, наблюдалось увеличение числа CD68-позитивных клеток в образцах висцерального жира и повышение экспрессии гена *CD68*.

Заключение. При экспериментальном диет-индуцированном МС жировая ткань приобретает провоспалительную активность за счет гипертрофии адипоцитов, инфильтрации иммунокомпетентными клетками (CD68⁺-макрофагами) и повышенной секреции провоспалительных хемо- и адипокинов (TNF- α , IL-6, MCP-1, лептина).

Ключевые слова: метаболический синдром, ожирение, воспаление, цитокины, макрофаги

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INTRODUCTION

To date, the problem of metabolic syndrome (MetS) and obesity has become a global medical and social issue and remains relevant due to the rapid increase in the prevalence of these pathological conditions worldwide. Diet-induced obesity is the most common form of obesity and is of particular interest from a pathogenetic viewpoint, as excessive calorie intake leads not only to lipid accumulation and disruption of all types of metabolism but also to the activation of inflammatory reactions in adipocytes and cells of the stromal – vascular fraction of adipose tissue [1, 2].

The steady increase in the number of overweight and obese people requires close attention from the scientific community. Despite significant progress in studying the clinical and laboratory manifestations of metabolic disorders, some aspects of the pathogenesis of MetS and obesity require further research. In particular, the molecular genetic mechanisms underlying adipose tissue dysfunction and mediating its role in the initiation of systemic metabolic disorders and chronic inflammation are still not fully understood [3, 4]. A priority remains research on the molecular basis of intercellular communication in adipose tissue, including key signaling pathways involving adipokines, cytokines, and extracellular vesicles, along with the epigenetic mechanisms that regulate these processes [5, 6].

Since pathological remodeling of adipose tissue, characterized by both structural and functional impairments, is a key pathogenetic factor in obesity and its associated metabolic disorders and systemic complications, it is crucial to study the mechanisms underlying the dysfunction of adipose tissue cellular components to understand their role in the pathogenesis of meta-inflammation in obesity.

For this purpose, experimental animal models serve as an indispensable tool, as they allow not only for the recreation of the pathophysiological features of MetS and obesity under controlled conditions but also for a detailed investigation of the relationship between the metabolic and inflammatory processes developing in visceral adipose tissue [7, 8].

The aim of the study was to investigate the parameters of proinflammatory activity of adipose tissue in an experimental model of MetS in rats.

MATERIALS AND METHODS

The study was performed on 24 male Wistar rats. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) (Minutes

No. 1 dated March 6, 2024). The procedures for animal care, housing, euthanasia, and experimental manipulations complied with international guidelines for good laboratory practice. The method for modeling MetS was described in detail by us previously [9]. The control group rats ($n = 12$) received a standard diet (protein:fat:carbohydrate 24%:6%:44%) with free access to food and water. The experimental group rats ($n = 12$) were fed with a high-fat, high-carbohydrate diet (HFHCD) (protein:fat:carbohydrate 16%:21%:54%) and provided with a 20% fructose drinking solution for 12 weeks.

Animals were euthanized by CO₂ asphyxiation. The blood samples were collected from the heart to obtain serum, and fragments of the white adipose tissue (WAT) were extracted. The relative mass of WAT (tissue mass/100 g of rat body weight) was assessed. The serum concentrations of glucose, triacylglycerides (TAG), and total cholesterol (TC) (all assays from Olvex Diagnosticum, Russia) were determined by enzymatic methods. Insulin (Rat INS(Insulin) ELISA Kit, Elabscience, China) and leptin (Rat Leptin ELISA Kit, ELK Biotechnology, China) levels were measured by enzyme-linked immunosorbent assay (ELISA). The HOMA-IR index (Homeostasis model assessment of insulin resistance) was calculated as (serum insulin) × (serum glucose) / 22.5.

To assess the secretory activity of WAT cellular elements, fragments of visceral fat were cultured in a nutrient medium based on DMEM/F-12 (Servicebio, China) supplemented with a penicillin – streptomycin antibiotic mixture (Capricorn Scientific, Germany), 200mM L-glutamine (Capricorn Scientific, Germany), and 2% bovine serum albumin (Sigma-Aldrich, USA). The dissection and preparation of adipose tissue samples were performed under sterile conditions; fat fragments weighing 150 mg were placed into the wells of a 24-well culture plate containing 1.5 ml of the nutrient medium. The samples were incubated for 24 hours in a CO₂ incubator at a temperature of 37 °C and 5% CO₂. Upon completion of the incubation, aliquots of the conditioned medium were taken and frozen at –70 °C. The concentration of adipocyte hormones (leptin and adiponectin) and cytokines: tumor necrosis factor-alpha (TNF α), interleukins (IL) (IL-6 and IL-10), and monocyte chemoattractant factor-1 (MCP-1) in the conditioned medium was determined by (ELISA) using the corresponding Rat ELISA Kit sets (ELK Biotechnology, China).

A pathomorphological study of WAT fragments was performed to analyze and assess the severity of morphological changes. For this purpose, during the sampling stage, the fat samples were fixed in 10% neutral buffered formalin (BioVitrum, Russia). To prepare histological specimens, the samples were washed for 24 hours to remove the fixative, then dehydrated in an isopropanol-based solution according to the manufacturer's protocol (IsoPrep, BioVitrum, Russia), and placed in an intermediate medium vaseline oil/liquid paraffin (MEDKHIM, Russia). The tissue samples were embedded in the paraffin medium Histomix (BioVitrum, Russia), and thin sections (5–6 μm) were made using an HM355 S automatic microtome (Thermo Scientific, USA). The sections were stained with hematoxylin and eosin (BioVitrum, Russia).

The presence of CD68+ cells in the adipose tissue was detected using an immunohistochemical method. Thin sections of the adipose tissue were deparaffinized in xylene and rinsed sequentially in 96% and 70% ethanol and then in distilled water. Subsequently, antigen retrieval was performed in a retrieval buffer (Target Retrieval Solution, pH 9.0, Dako, USA) using a water bath at 96°C. After cooling, the sections were sequentially treated with a peroxidase block (Peroxidase-Blocking Reagent, Dako, USA) and incubated for 45 minutes with the primary antibody (PA5-78996, Invitrogen, Thermo Fisher Scientific, USA) at a 1:100 dilution under the

conditions recommended by the manufacturer. The samples were then washed twice with phosphate buffer and incubated for 30 minutes with a secondary antibody (Rabbit IgG (H+L), HRP, 31460, Invitrogen, Thermo Fisher Scientific, USA). The EnVision FLEX system (Dako, USA) was used for visualization, with diaminobenzidine solution (Liquid DAB+, Dako, USA) employed as the chromogenic substrate; the nuclei were counterstained with hematoxylin (BioVitrum, Russia). Cell counting was performed in 30 fields of view for each sample, obtained using the EVOS M7000 system (Thermo Fisher Scientific, USA).

The level of *CD68* gene expression was determined. For this purpose, total mRNA was extracted from WAT fragments using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), followed by quantitative mRNA analysis on the Agilent 4150 TapeStation automated gel electrophoresis system (Agilent Technologies, USA). Complementary DNA (cDNA) was then synthesized from the mRNA template using the M-MuLV-RH reverse transcriptase kit (Biolabmix, Russia). Quantitative real-time polymerase chain reaction (qPCR) was performed using the BioMaster HS-qPCR (2 \times) kit (Biolabmix, Russia). The analysis was carried out using the $2^{-\Delta\Delta C_t}$ method relative to the expression level of the housekeeping gene β -actin (*ACTB*). Primers and probes for the *ACTB* and *CD68* genes were synthesized by DNA-Sintez LLC (Russia) (Table 1).

Table 1

Sequences of Primers and Probes for the <i>ACTB</i> and <i>CD68</i> Genes		
Gene	Primer sequences (F&R)	Probe sequences (FAM/BHQ1)
<i>ACTB</i>	5' GAGAAGATGACCCAGATCATGTT 3' 5' ATAGCACAGCCTGGATAGCAA 3'	5' AGACCTCAACACCCCAGCCAT 3'
<i>CD68</i>	5' GACACTTCGGGCCATGCT 3' 5' TAACGCAGAAGGCAATGAG 3'	5' CCAATCTCTCTTGCTGCCTCTCATC 3'

Statistical analysis was performed using SPSS Statistics 23 software. Data consistent with a normal distribution are described as mean and standard deviation ($M \pm SD$), while data not consistent with a normal distribution were presented as median and interquartile range ($Me (Q_{25}; Q_{75})$). Difference analysis was conducted using the Student's t-test or the Mann–Whitney *U*-test. Differences were considered statistically significant at $p < 0.05$. To assess the relationship between parameters, the Spearman's rank correlation coefficient (r) was determined.

RESULTS

It was found that the animals in the experimental group exhibited an increase in body weight (485.1 ± 33.8 g vs. 430.3 ± 25.4 g in the control group ($p = 0.005$)), as well as in the relative mass of visceral fat, which was 4.6 ± 0.5 g in the experimental group and 2.1 ± 0.3 g in the control group ($p = 0.002$).

In the experimental group of rats subjected to the 12-week HFHCD, statistically significant changes in blood biochemical parameters were observed

compared to the corresponding values in the control group animals. Specifically, there was an increase in the concentration of glucose and TAG (on average, 1.4-fold, $p < 0.05$) and cholesterol of more than 1.6-fold ($p = 0.009$). These detected changes were accompanied by a marked increase in blood levels of insulin and leptin (Table 2). It was found that in rats fed with the HFHCD, a positive correlation was established between the increase in adipose tissue mass and the increase in serum concentrations of leptin ($r = 0.57$, $p = 0.005$), glucose ($r = 0.62$, $p < 0.001$), and insulin ($r = 0.61$, $p < 0.001$).

Table 2

Biochemical Parameters of Rats fed a Standard diet or HFHCD, $M \pm SD$			
Parameter	Control group ($n = 12$)	Experimental group ($n = 12$)	p
Glucose, mM	5.1±0.5	6.6±0.2	0.005
Insulin, pM	12.9±0.7	22.3±5.9	0.002
Leptin, ng/ml	2.2±0.2	6.9±1.3	0.003
HOMA-IR	2.9±0.2	6.5±0.4	0.012
Triacylglycerols, mM	1.8±0.3	2.6±0.4	0.014
Total cholesterol, mM	2.9±0.3	4.8±0.4	0.009

Here and in Tables 3, 4: p – level of statistical significance of the differences.

According to ELISA of conditioned medium samples from cultured WAT fragments, it was found that in the experimental group of rats, the concentration of proinflammatory cytokines IL-6, TNF α , and MCP-1 exceeded that in the control group, whereas the level of anti-inflammatory IL-10, on the contrary, was lower than the control values (Table 3). Along with this, the samples from the experimental animal group showed a statistically significant increase in leptin concentration (on average 3-fold, $p = 0.004$), but a decrease in adiponectin concentration (on average 1.4-fold, $p = 0.018$).

Histological examination of WAT sections revealed that in the control group rats, the WAT consisted of large lobules composed of large adipocytes of regular rounded shape and

approximately uniform size; the connective tissue interlobular septa were poorly developed, contained blood vessels of normal structure, and showed no signs of congestion (Fig. 1, A). In the animals of the experimental group, the adipose tissue also has a lobular structure. However, the lobules were smaller than those in the control group animals, were irregularly rounded in shape, and the adipocyte size varied significantly. The interlobular connective tissue septa were more pronounced, with areas of vascular congestion (engorgement of blood vessels) observed within them (Fig. 1, B). Thus, the adipose tissue of the rats in the experimental group exhibited pathomorphological signs of impaired circulation, hypertrophy, and hyperplasia of fat cells.

According to the immunohistochemical analysis results (Fig. 2, A), it was found that the experimental group showed an increase in the number of cells with a CD68 phenotype compared to the control: 158.6 (142.3; 178.1) vs. 52.4 (45; 62.5) ($p = 0.001$).

A comparative assessment of the *CD68* gene expression level by cellular elements of the adipose tissue (Fig. 2, B) revealed an approximately 2-fold increase in its level in the samples from the experimental group animals compared to the control samples: 1.6 (0.9; 2.5) vs. 4.6 (2.7; 5.8) relative units ($p = 0.001$).

Correlation analysis revealed a statistically significant positive association between the concentrations of cytokines IL-6, TNF α , MCP-1 and the level of *CD68* gene expression in rat adipose tissue (Table 4).

Along with this, it was established that an increase in the concentration of leptin from adipose tissue samples in the conditioned medium positively correlated with the level of produced pro-inflammatory cytokines; whereas for adiponectin, an inverse correlation with the same cytokines was revealed (Table 4). A positive relationship was also found between an increase in the *CD68* gene expression level and the concentration of leptin ($r = 0.58$, $p = 0.001$) and serum insulin in rats with MetS ($r = 0.65$, $p = 0.001$).

Table 3

Concentration of Adipocyte Hormones and Cytokines in the Conditioned Medium of WAT, $Me (Q_{25}; Q_{75})$			
Parameter	Control group ($n = 12$)	Experimental group ($n = 12$)	p
Leptin, ng/ml	0.3 (0.1;0.5)	0.9 (0.7;1.1)	0.024
Adiponectin, ng/ml	8.4 (7.9;8.8)	6.1 (5.8;6.9)	0.018
IL-6, pg/ml	107.5 (100.4;129.7)	169.57 (148.4;198.2)	0.017
IL-10, pg/ml	119.8 (90.7;135.8)	84.5 (69.2;100.6)	0.021
TNF α , pg/ml	0.9 (0.5;1.2)	2.3 (1.9;2.8)	0.003
MCP-1, pg/ml	135.9 (110.6;176.6)	272.7 (161.6;309.1)	0.005

Table 4

Spearman's Rank Correlation Coefficients (R) between WAT Secretory Activity Parameters and CD68 Gene Expression Level in Rat Adipose Tissue				
Parameters	Concentration of cytokines, pg/ml			
	IL-6	IL-10	TNF-α	MCP-1
Concentration of leptin, ng/ml	0.61, <i>p</i> = 0.001	-0.32, <i>p</i> = 0.006	0.58, <i>p</i> = 0.001	0.55, <i>p</i> = 0.001
Concentration of adiponectin, ng/ml	-0.41, <i>p</i> = 0.003	0.51, <i>p</i> = 0.001	-0.48, <i>p</i> = 0.002	-0.53, <i>p</i> = 0.001
CD68 gene mRNA expression, r.u.	0.65, <i>p</i> = 0.001	-0.29, <i>p</i> = 0.008	0.51, <i>p</i> = 0.002	0.64, <i>p</i> = 0.001

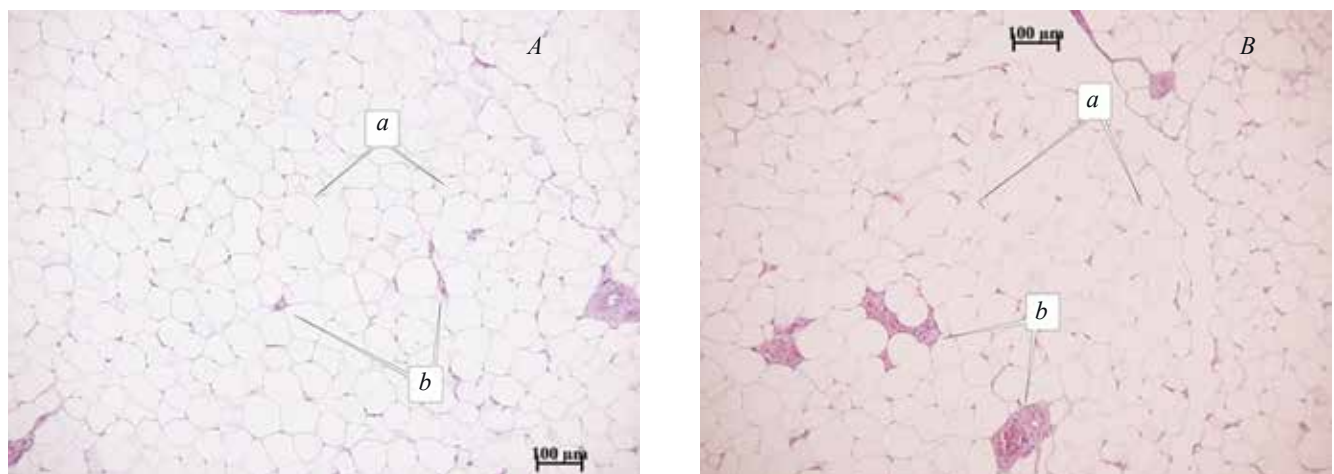


Fig. 1. Morphological features of visceral adipose tissue: A – fragment of white adipose tissue from a rat in the control group. Adipocytes (a) and blood vessels (b), ×200. B – fragment of white adipose tissue from a rat in the experimental group. Anisocytosis of adipocytes (a) and venous vessel congestion (b), ×200.

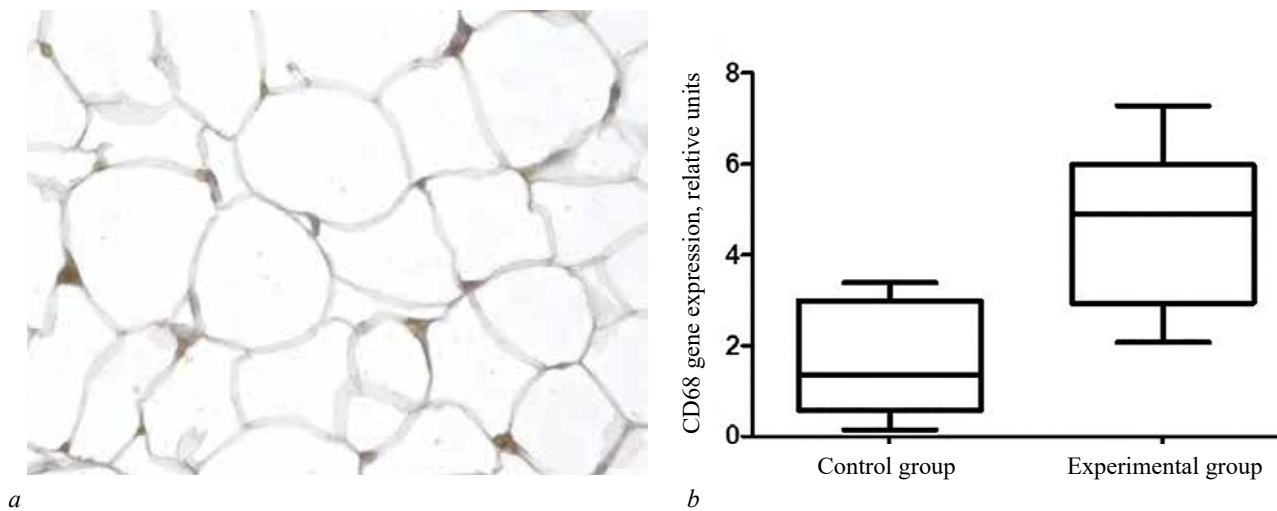


Fig. 2. Evaluation of CD68 expression by adipose tissue cells: a – immunohistochemical reaction for CD68-positive cells in a white adipose tissue sample of a rat from the experimental group. Diaminobenzidine and hematoxylin counterstain. ×400; b – Relative CD68 mRNA expression in control and experimental samples of rat adipose tissue; *p* – level of statistical significance of differences.

DISCUSSION

A number of studies have established that lipid overload in adipose tissue leads to structural and functional alterations. These are characterized not only by adipocyte hypertrophy but also by excessive

accumulation of the extracellular matrix due to increased activity of collagen-producing fibroblasts. This results in a significant reduction in tissue elasticity, impaired blood supply, and the development of hypoxia and oxidative stress reactions [10, 11]. The results of our own pathomorphological study of adipose

tissue samples confirm these findings. Specifically, the animals in the experimental group exhibited adipocyte hypertrophy and anisocytosis, impaired blood supply, engorgement of venous vessels, and thickening of the interlobular connective tissue layer.

Excessive secretion of pro-inflammatory adipocyte hormones (leptin, visfatin, and chemerin) and chemokines (MCP-1, chemokine (C-X-C motif) ligand 12 (CXCL12)) by hypertrophied adipocytes contributes to the initiation and maintenance of inflammation [3, 12]. Our studies have shown that the increase in adipose tissue mass in animals fed with a HFHCD for 12 weeks correlates with elevated serum concentrations of leptin and insulin. Furthermore, the leptin level was statistically significantly increased in the conditioned medium of the rat WAT from the experimental group. Leptin, which is overproduced by hypertrophied adipocytes, is known to be actively involved in the pathogenesis of insulin resistance through the activation of pro-inflammatory cytokine (TNF α and IL-6) production, the enhancement of oxidative stress reactions, and a direct effect on insulin-secreting cells [13]. Thus, a vicious cycle emerges – leptin resistance and insulin resistance mutually reinforce each other, contributing to the progression of metabolic disorders.

The inflammation of adipose tissue developing in this context is characterized by increased infiltration by immune cells (macrophages and lymphocytes) and the secretion of inflammatory mediators – reactive oxygen species, cytokines, chemokines, non-enzymatic cationic proteins, etc. [14]. Our study demonstrated that in the experimental group of rats, the production of pro-inflammatory cytokines IL-6, TNF α , and MCP-1 by adipose tissue cells increased, while the level of the anti-inflammatory cytokine IL-10 decreased.

The shift in the functional profile of resident and recruited macrophages from the M2 to the M1 phenotype, which is characterized by increased secretion of the pro-inflammatory cytokines TNF α , IL-6, and IL-1 β , contributes to the enhancement of pro-inflammatory activity in adipose tissue [15]. It is noted that macrophages expressing the CD68 surface marker play a key role as the primary cellular component of adipose tissue, regulating the inflammatory response and modulating the secretion and differentiation of various cytokines [16, 17]. Moreover, their quantity is positively correlated with the insulin resistance index. At the same time, a study of the adipocyte microenvironment in obesity revealed a “crown-like structure” zone containing M1-polarized CD68+

macrophages and CD8+ T-lymphocytes [18]. We demonstrated that the animals in the experimental group exhibited an increase in the number of CD68-positive cells in the visceral adipose tissue samples, which is supported by the upregulation of the *CD68* gene. Furthermore, the active production of pro-inflammatory cytokines (IL-6, TNF α , and MCP-1) and leptin by visceral adipose tissue cells in rats with MetS directly correlates with the level of *CD68* gene expression. As established, IL-6 stimulates monocyte migration into adipose tissue by increasing the expression of adhesion molecules (VCAM-1/ICAM-1) and enhances macrophage polarization towards the M1 phenotype [19], while MCP-1 is a key chemokine that recruits monocytes through the activation of the CCR2 receptor [20]. Leptin, acting through the Ob-Rb receptor and the JAK2/STAT3 signaling pathway, directly stimulates the proliferation of CD68+ cells in visceral fat [21]. Our study also revealed a positive correlation between the increased *CD68* gene expression level and the rising concentrations of leptin and insulin in the serum of rats with MetS.

As mentioned, T-lymphocytes are also actively involved in the inflammatory response, particularly cytotoxic cells (CD8+) and T-helper 17 cells (Th17), whereas the tissue pool of regulatory T-lymphocytes (Treg), on the contrary, becomes depleted [22, 23]. One of the mechanisms driving the progression of inflammation in adipose tissue is the activation of the NLRP3 inflammasome complex (NLR family pyrin domain containing 3) in macrophages. This activation mediates the increased production of pro-inflammatory cytokines IL-1 β and IL-18, stimulates pyroptosis of adipose tissue cells, and enhances fibrosis due to increased production of transforming growth factor beta (TGF β) [24]. Studying the transition of macrophages from the anti-inflammatory phenotype (M2) to the proinflammatory phenotype (M1) in the context of adipose tissue inflammation, particularly through the modulation of cytokine secretion alongside the activation of the JNK/NF- κ B signaling pathway and the NLRP3 inflammasome complex, is crucial for understanding the pathogenesis mechanisms of MetS and obesity.

CONCLUSION

Thus, the study found that in experimental diet-induced MetS, adipose tissue acquires pro-inflammatory activity through adipocyte hypertrophy, infiltration by CD68+ macrophages, and increased secretion of proinflammatory chemokines and

adipokines (TNF α , IL-6, MCP-1, and leptin). All of this contributes to the development and maintenance of chronic systemic inflammation, insulin resistance, and exacerbates metabolic disorders. Undoubtedly, a detailed study of the molecular and cellular mechanisms of adipose tissue dysfunction in MetS and obesity is of key importance for understanding the pathogenesis of these conditions and for developing new diagnostic methods and personalized treatment approaches for their associated complications.

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