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HIGH LIPID DIET MODEL AND THE EXPRESSION OF INTERLEUKIN-4

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ABSTRACT

PURPOSE: The aim of the present study was to demonstrate the expression of inflammatory marker -IL-4 in visceral adipose tissue, testis, and blood serum when experimentally presented for obesity in rats.

METHODS: We used a high-lipid diet model leading to the development of obesity in rats. 50 male Wistar rats were used. They were divided into two groups - control group (C) - fed with standard rodent food - experimental group (E) - fed high lipid diet for a period of 4 weeks. Blood, visceral adipose tissue and testis were collected after the end of the experimental period. For the determination of IL-4 in the testis and visceral adipose tissue, we used the immunohistochemical method and in the blood serum the ELISA method.

RESULTS: *We detected that IL-4 is increased in the control group* blood serum, adipose tissue, testis *(the seminiferous tubules and the interstitial cells) of the control group compared to the experimental group.*

CONCLUSION: *The study shows that obesity leads to the generation of complex and multilayered interactions between adipocytes, IL-4 and germ cells with adverse effects on the* germinal epithelium of the testicle.

Key words: Animal model of obesity, low-grade inflammation, interleukin -4, adipose tissue, testis.

INTRODUCTION

Nowadays, despite the continual improvement in living conditions, the global health of the population is showing an increase in overall morbidity. The percentage of infertile couples is also on the rise (1). This necessitates the search for the causes of these results precisely in the way of life and nutrition as the main factor regulating the processes in the body(2). Obesity in men has a negative effect on overall health and fertility (3).

The quality of sperm counts has been steadily declining, which has necessitated a change in the reference values several times in recent decades (4). The influence of genetic, immune and endocrine factors, accompanied by infections, varicocele are responsible for between 30 to 40% of the causes of male infertility (4). More than 60% of infertility is defined as idiopathic infertility for no apparent reason (5). These anomalies represent one of the most prevalent group of patients with reduced volume, concentration, and morphology, diagnosed as Oligo-Asteno-Teratozoospermia (OAT) (5), which are not associated with other disorders in men. The causes of these disorders can be of many different types, such as stress, alcohol, cigarettes, medications, environmental factors, eating habits and accompanying disorders affecting a man's reproductive function (6, 7).

Consumption of trans-fat rich and antioxidant poor food is associated with low-grade chronic inflammation (8). Low-grade inflammation causes disorders of fat metabolism and related diseases such as obesity and diabetes (9).

The growth of white adipose tissue is due to an increase in the volume and hypertrophy of adipocytes, accompanied by changes in their functionality, and normal homeostasis (10).

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In this way, adipocyte hypertrophy is likely to impair their function through inflammation-dependent mechanisms, disrupting insulin resistance and energy metabolism (11).

Adipocytes secrete a large number of signaling pro-inflammatory proteins and interleukins, further stimulating the inflammatory processes (12).

Interleukin-4 (IL-4) is one of the major inflammatory cytokines that activates the Th2-dependent immune response and has been shown to increase in low-grade chronic inflammation (13).

The purpose of this study is to determine the presence of IL-4 in visceral adipose tissue and structures of the testis by the model of high-lipid diet and the possible effect on spermatogenesis.

MATERIAL AND METHODS

Animals

Sixty, eight-weeks-old male Wistar rats (weight 130 – 180 g) were obtained from University Vivarium. They were randomly divided into two groups, 12 animals in each:
Control group (C), fed with standard rodent food (D12450H, Research Diets, Inc. – contain: proteins - 19.2 g%, carbohydrates - 67.3 kg%, fat - 4.3 g% and energy value of 3.85 kcal / g.) - for 14 weeks. Experimental group (E), fed with a high-fat diet (D12451 – Research Diets, Inc.- contain: proteins - 24 g%, carbohydrates - 41 g%, fat - 24 g%, energy value 4.73 kcal / g.) - for 14 weeks.

Blood serum were collected and immediately frozen at -18ºC, tissue samples from testis and adipose tissue were fixed in 10% formalin for imunohistochemistry.

Immunohistochemistry

Sections of the testes and adipose tissue thickness 5μm were deparaffinized, then subjected to antigenic detection of the epitopes with citrate buffer and an endogenous peroxidase blockade was made with hydrogen peroxidase 3%, kit (ref: № BBK 120, Scy Tek, USA) was used to block the endogenous biotin and reagent to block non-specific binding (Superblock, Scy Tek) follows incubate for 24 hours (at 4 ° C) with monoclonal mouse anti-IL-4 - 1:150 (Dako, Denmark), next incubated with secondary antibody: biotinylated anti-rabbit for 10 min. at room temperature.

The reaction was visualized with 3,3`-diaminobenzidine tetrachloride (DAB, ScyTek Lab. Inc., USA), and counterstaining with Mayer's hematoxylin. The preparations were observed with a light microscope at magnification x 200 and x 400.

Analysis of immunohistochemical reactions

The analysis was performed on sections from testis of Wistar rats (n = 6 for each group of animals - control and fed with high lipid diet). Changes in expression of the investigated markers in terms of their location and intensity are evaluated. The intensity of the reaction is determined on a semi-quantitative scale, reflecting the positivity of a certain number of cells from 100 counts ("+" - poor staining in single cells, "2 +" - presence of expression in 50% of the counted cells, "3 +" - intense homogeneous expression in 100% of cells).

Statistical analyses

The data obtained were expressed as the mean ± standard error of the mean (SEM). The number of tissue preparations used in each experiment is indicated by n. Statistical differences were Student's t-test, $p < 0.05$.

Ethical approval

All methods used in the study were approved by the Scientific Ethics Committee at the Medical University of Plovdiv with resolution №P1041/25.04.2017 and Bulgarian Agency for Food Safety (BAFS – resolution №55/23.06.2016).

Results for IL-4

IL-4 levels in the blood serum of rats fed a high-lipid diet showed a slight increase compared to the control group. This increase did not show a statistically clear difference, $p = 0.304$.

Table 1. *Quantitative changes in the concentrations of IL-4 in experimentally induced obesity.*

	n	Median	Mean ±SD	95% CI	p-value, „E" against „C"
IL-4 pg/ml,K - serum	25	26.093	29.739 ±9.391	23.022-36.457	
IL-4 pg/ml, E - serum	25	28.345	25.560 ±8.234	19.670-31.450	>0.05

* C - control group included animals fed for 14 weeks with D12450H; The E (obese) experimental group covered animals fed for 14 weeks with D12451

Production of IL-4

We used an immunohistochemically stains to determine the level of expression of our IL-4 markers in the testis and adipose tissue. Expression of IL-4 was determined adipose tissue shows a clear upward trend. Control cells positively + (++), but in the experimental group, the number of positive cells increased (+++). In the experimental group, we found an increase in the size of adipocytes expressed in hyperplasia and hypertrophy compared to control animals. These findings suggest that IL-4 is involved in lipid deposition and adipocyte size increases.

In the testis control group, we found that the number of IL-4 positive cells visualized in seminiferous tubule *(+++) and interstitial cells (+++) was significantly higher compared to experimental animals (*seminiferous tubule *(++) and interstitial cells (++) in the testis.*

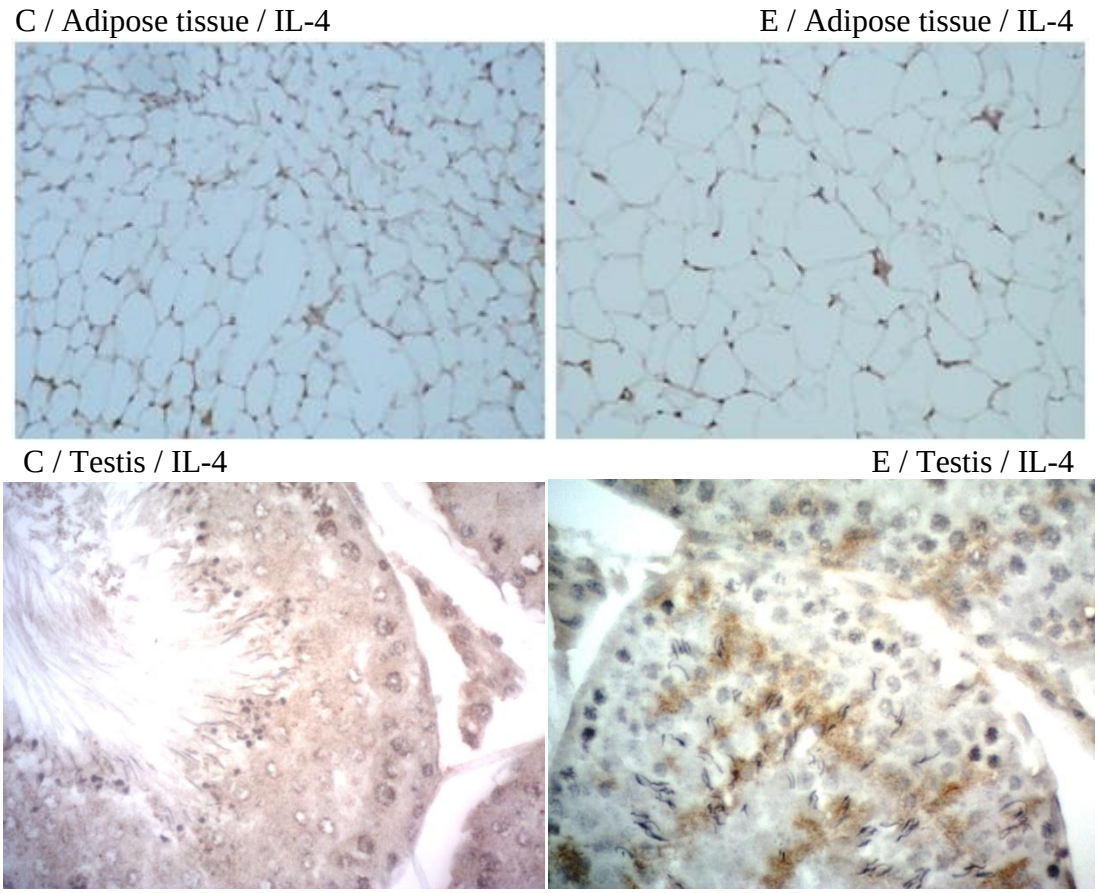

Figure 1. Control (C) and experimental groups (E): The adipose tissue, we found increased IL-4 expression and adipocyte hyperplasia in the experimental comparison with the control group, magnification ×200; IL-4- higher expression in the C group compared to E in the testis; magnification, x400.

Table 2. *Comparisons data of the measured immunoreactivity*

Immunohisto-chemistry	C		E		C	E
	Testis/germ cells	Testis/Leydig cells	Testis/germ cells	Testis/Leydig cells	Adipose tissue	
IL-4	+++	+++	++	++	++	+++

Control (C) and experimental groups (E). Ratio of immunohistochemistry positive cells fo IL-4 in the testis and adipose tissue.

DISCUSSION

The continuous increase in the rate of obesity in men requires a better understanding of the mechanism affecting the individual systems and in particular the reproductive tract in men (14).

The effect of obesity on male fertility is thought to be multifactorial and may be modulated by genetic, endocrinal and environmental influences (4-15).

The analysis of in vitro fertilization data, as well as animal models, has shown that obesity in male individuals has been inherited for generations, through a decrease in sperm quality and poor implantation of embryos. These changes affect the development of the embryo and predispose to diabetes and chronic diseases of the individual in the course of his life (16-17).

In this study, we track the effects of a high-lipid diet, obesity, and pronounced immune response through changes in the levels of IL4.
We found that the body weight of experimental animals fed a high lipid diet was significantly higher than the control group. The results of our comparative analysis of white adipose tissue indicate adipocyte hypertrophy in the experimental group. These results indicate that adipocyte obesity and growth in white adipose tissue lead to an increase in adipocyte volume and aging, and similar results have been reported by Chawla et al. (18). Hypertrophic adipocytes have reduced insulin sensitivity (19), they turn into old, necrosing adipocytes activating pro-inflammatory phenotype macrophages (20-21). Macrophages found in adipose tissue secrete pro-inflammatory cytokines, with their increased level inhibiting differentiation and stimulating the aging of adipocytes (22).

Previous studies have shown that the obesity-induced, low-grade inflammation is characterized with much lower levels of circulating cytokines compared with acute inflammation (23).

The levels of the inflammatory markers, measured in blood serum concentration correlate with the body mass index (24).

Our analysis of the expression of IL-4 in visceral adipose tissue showed an increase in the experimental group. IL-4 is associated with the lipid deposition in adipocytes and increases the secretion of inflammatory factors that underlie low-grade inflammation of adipose tissue (25).

Interleukin-4 (IL-4) is one of the main inflammatory cytokines that activates Th2-dependent immune response and its increased expression was observed in the course of diseases characterized as obesity, mainly via inducing M2-like macrophages polarization (26).

The results of IL-4 expression in the testis showed a decreased expression in the testicular interstitium, as well as in the germ cells in the seminiferous tubules in the experimental compared to the control group.

From the data on the expression of IL-4 in the testis, we can assume that in the structures of the testis, it is more likely involved in spermatogenesis and maturation of sperm. Many studies have shown that somatic testicular cells (Sertoli, Leydig, peritubular cells) under physiological conditions produce large amounts of cytokines such as interleukin 1 (IL-1) and IL-6, participating in its function and regulation of the blood-testicular barrier (27-28).

Large numbers of cytokines such as IL-1α, IL-1β, IL-2, IL-4 - IL-8, IL-10 - IL-13, IL-17 and IL-18, chemokines, and growth factors, interleukins, transforming growth factor (TGF), macrophages and inflammatory proteins are isolated from seminal plasma in man as natural components (29-30).

The testis is an immune-fit organ and the presence of a large number of mediators with dual inflammatory and anti-inflammatory functions is accepted by many authors as part of the maintenance balance necessary for its functioning (31).

An increase in values could be changed due to infections and inflammation causes a change in immune homeostasis, which leads to male infertility.

It is undisputed that obesity in men has a negative effect on infertility through changes in hormonal levels as well as through direct changes in the quality of sperm performance (32).

Many studies have shown that obesity in men is associated with insulin resistance, as the high levels of insulin suppress testosterone production (28) due to disorders in the pituitary-testis axis, affecting spermatogenesis in these patients (33-34).

The increased concentration of pro- and anti-inflammatory markers in the spermatogenic epithelium and testicular interstitium inevitably leads to a change in its functionality. Reduced

testosterone production has a negative effect on immunosuppression while maintaining immune tolerance in the testis and leads to a decrease in sperm concentration, motility and sperm count (35-36).

The increased concentration of pro-inflammatory and anti-inflammatory markers in the spermatogenic epithelium and testicular interstitium inevitably leads to a change in its functionality. Reduced testosterone production has a negative effect on immunosuppression while maintaining immune tolerance in the testis and leads to a decrease in sperm concentration, motility and sperm count (35-36).

In conclusion, the study shows that obesity leads to the generation of complex and multilayered interactions between adipocytes, IL-4 and germ cells, with adverse effects on the sperm epithelium. Chronic inflammation stimulates the invasion of pro-inflammatory markers in the testis, altering the immunosuppressive microenvironment. These disorders impair the quality of semen parameters and are one of the possible causes of idiopathic infertility in men.

Further studies are needed to clarify the role of the immune cells in lipid metabolism, and in particular on the role of IL-4 in cytokine-immune interactions, insulin sensitivity and metabolism.

The new knowledge would contribute to the advancement of the diagnosis and treatment of male infertility.

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