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Original Contribution

POTENTIAL PRO-INFLAMMATORY EFFECTS OF HIGH-CALORIE DIETS MEASURED BY BLOOD NEUTROPHIL H_2O_2 PRODUCTION

K. Trifonova, P. Dzhelebov*

Department of General and Clinical Pathology, Faculty of Veterinary Medicine,
Trakia University, Stara Zagora, Bulgaria

ABSTRACT

PURPOSE: The aim of the study was to evaluate the potential pro-inflammatory effects of different high-calorie diets in a rat experimental model. METHODS: A total of 56 male Wistar rats were included in the experiment. Animals were divided into seven equal groups – one pool group, in which tested indicators were evaluated before the start of the dietary regimens, and six more groups of rats, which were fed different diets for a period of five weeks – standard diet (SD), high-fat diet (HFD), high-carbohydrate diet (HCHD), high-fat high-carbohydrate diet (HFHCHD), standard diet plus E960/RA60 (SDRA) and high-fat diet plus E960/RA60 (HFDRA). Total white blood cell count and differential were measured using an automated analyzer. H_2O_2 production by blood neutrophils was measured using the nitroblue tetrazolium reduction test on a blood smear. RESULTS: Results demonstrate that some of the high-calorie diets, which contain sucrose, significantly increase H_2O_2 production by blood neutrophils. CONCLUSIONS: Diets with high content of sucrose may have the potential to induce cellular damage and subsequent inflammatory response in certain tissues.

Keywords: high-fat diet, high-carbohydrate diet, reactive oxygen species, neutrophil, inflammation

INTRODUCTION

The causes of insulin resistance and diabetes type 2 include factors of various nature, such as lack of physical activity, obesity, environmental factors, genetic factors, dietary factors (increased calorie intake), hormonal disorders (Cushing's syndrome; acromegaly) and certain medications.

Many theories have tried to explain the molecular mechanisms for the onset and development of insulin resistance (1, 2). All factors that influence glucose control and production of insulin and counterregulatory hormones, as well as target cells and tissues, seem to be involved (3). Insulin resistance often results from obesity. Consumption of foodstuff that has high glycemic load is usually considered to be predisposing factor for obesity (4). Diets with high fat content also lead to excessive lipid accumulation (5). Obese individuals have an excess of body fat, which in turn establishes a state of low-grade inflammation through the secretion of certain cytokines (6). Pro-inflammatory cytokines are produced mainly by phagocytic cells of the immune system and cells of adipose tissue, especially in abdominal obesity (7). Obesity-induced inflammation has the potential to make adipose tissue, skeletal muscle and liver become insensitive to insulin by inhibiting insulin signal transduction. Pro-inflammatory cytokines and nitric oxide seem to play essential role in disrupting the function of insulin target cells (8). In addition to this, reactive oxygen species produced by mitochondria of adipocytes or by nicotinamide adenine dinucleotide phosphate oxidases of adipocytes or certain immune cells also contribute to the onset and progression of insulin insensitivity (9). Macrophages and

***Correspondence to**: *P. Dzhelebov, Department of General and Clinical Pathology, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, Bulgaria, tel.: +359 42 699 581, E-mail: petko.dzhelebov@trakia-uni.bg*

eosinophils are the cells suspected to contribute to insulin resistance and low-grade inflammation (10). Platelets also seem to be involved in the pathogenesis of metabolic disorders, as some studies have found that platelet count is positively linked to insulin resistance (11). Some studies have even found relation between insulin resistance and membrane receptors and fluidity, which can be significantly influenced by cellular metabolism and metabolic rate (12, 13).

Obviously accumulation of excess body fat, resulting from high energy intake supplied by glucose or free fatty acids, is associated with a state of low-grade inflammation and insulin resistance. Thus, the aim of the present study was to evaluate the potential pro-inflammatory effects of high-calorie diets in an *in vivo* experimental rat model, measured by the total white blood cell count and differential and the ROS generation by blood neutrophils.

MATERIALS AND METHODS

Animals

A total of 56 Wistar male rats, at around 9 weeks of age, were included in the study. Rats were kept in cages located indoors. Optimal environmental conditions were provided – temperature of 22 ± 2ºC, humidity – 55 ± 10 %, 12:12 h light-dark cycle. Animals had free access to feed and water. All *in vivo* procedures complied with the minimum ethical requirements for the protection and humane treatment of laboratory animals (Permit № 335/25.10.2022 of the Bulgarian Food Safety Agency).

Experimental design

Seven groups of rats were used in the experiment (n=8 per group) – one of the groups was used to measure the basal values of laboratory parameters before the start of the experimental dietary regimens and the rest six groups were fed different diets over a five-week period.

Groups of experimental animals: 1) BD group (before diet) – in this group laboratory parameters were measured before the start of the dietary regimens; 2) SD group (standard diet) – animals from this group were fed standard pelleted feed for laboratory rats (Melhran, LTD, Bulgaria); 3) HFD group (high-fat diet) – animals were fed the same standard pelleted feed enriched in lard (40% of the energy content coming from fat); 4) HCHD group (high-carbohydrate diet) – rats from this group received the standard pelleted feed supplemented with sucrose (75% of the energy intake was supplied by carbohydrates); 5) HFHCHD group (high-fat high-carbohydrate diet) – animals from this group received the standard pelleted feed enriched in both lard and sucrose (30% of calories supplied by fat and 57% by carbohydrates); 6) SDRA group (standard diet plus stevia extract - E960/RA60) – animals received the standard pelleted feed with added stevia extract (the taste equivalent of the sucrose in the high-carbohydrate diet, according to the manufacturer`s instructions (Balgarska Stevia, LTD, Bulgaria); this group served to test the potential positive effects of reducing the energy content of the high-carbohydrate diet by substituting sucrose with stevia extract; 7) HFDRA group (high-fat diet plus stevia extract - E960/RA60) – animals from this group received the standard pelleted feed enriched in lard (same amount as in high-fat high-carbohydrate diet) and stevia extract (the taste equivalent of sucrose in high-fat high-carbohydrate diet); this group served to evaluate the effects of reducing the energy content of the HFHCHD by substituting sucrose with stevia extract.

Contents of Stevia extract (Balgarska Stevia, LTD, Bulgaria): 96.8% of steviol glycosides as follows – 61.06 % rebaudioside A, 30.36 % stevioside, 12.42 % rebaudioside C, 2.49 % dulcoside A, 0.2 % steviolbioside, 0.16 % rebaudioside B, 0.12 % rebaudioside.

Blood sampling

Blood samples were taken after the completion of the 5-week dietary regimen for each group. Blood samples were obtained after overnight fasting by retro-orbital bleeding under general anesthesia (Xylazine – 8 mg/kg + Ketamine – 90 mg/kg; i.p.).

Laboratory parameters

Total white blood cell count and differential – WBC, Lym%, Mon%, Neu%, Bas%, Eos%, Lym#, Mon#, Neu#, Eos#, Bas#, were measured using an automated analyzer Mindray BC-5000 Vet (Mindray Medical International Limited).

Nitroblue tetrazolium reduction test (NBT-test) – Microscopic slide histochemical nitroblue tetrazolium chloride reduction test was performed to evaluate ROS (H_2O_2) production by neutrophils. *Principle:* H_2O_2 converts the colorless nitroblue tetrazolium (NBT) to a deep blue colored compound, which

is called NBT formazan. In active cells insoluble NBT formazan is visible as fine purple deposits **(Figure 1).** *Protocol:* In a siliconized tube 0.1 ml heparinized blood (100 IU/ml) is incubated with 0.1 ml 0.2% NBT solution (in phosphate buffer) for 15 min at 37^0C. Then incubation continues for another 15 min at room temperature. Smears are prepared (safranin staining). Microscopic observation is made under oil immersion. The number of ROS (H_2O_2) producing neutrophils is calculated as percentage of total neutrophil count (100) on the blood smear.

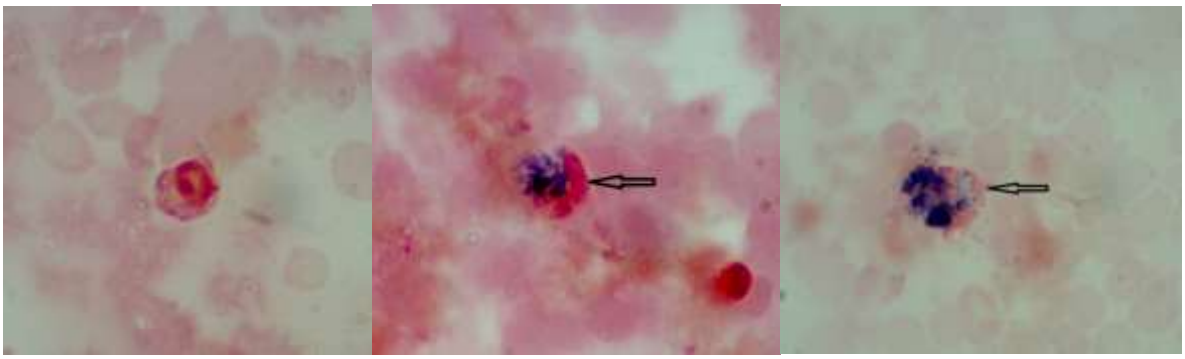

Figure 1. Neutrophils producing hydrogen peroxide (H_2O_2) contain deep blue colored NBT formazan (arrows). Safranin staining (100 x objective; 1000 x magnification).

Statistical analysis

Values of laboratory parameters are presented as mean ± standard deviation. Raw data was analyzed by one-way ANOVA test and Tukey`s post hoc test (Graph Pad InStat3). The level of statistically significant differences is $p<0.05$.

RESULTS

At the end of the experimental period no statistically significant differences were found in total white blood cell counts and differential except for eosinophils. Eosinophils (x10^9/L) increased significantly in groups SDRA (0.42±0.13) and HFDRA (0.42±0.15), as compared to BD group (0.18±0.08), ($p<0.01$). Eosinophils (%) increased also in groups SD (3.22±0.53; $p<0.01$), HCHD (3.01±0.89; $p<0.05$), SDRA (3.14±0.95; $p<0.01$) and HFDRA (2.99±0.72; $p<0.05$), as compared to BD group (1.73±0.36), **(Table 1).**

Table 1. *Total white blood cell count and differential in rats (n=8) before the start of the diet (BD) and in groups SD (n=8), HFD (n=8), HCHD (n=8), HFHCHD (n=8), SDRA (n=8), HFDRA (n=8). Results are presented as mean values ± SD.*

Group / Parameter	BD	SD	HFD	HCHD	HFHCHD	SDRA	HFDRA
WBC (x10^9/L)	9.17±1.8	9.81±1.85	11.52±2.34	10.6±1.77	12.27±2.13	12.43±2.11	12.38±0.52
Lym# (x10^9/L)	8.02±4.23	7.04±1.6	7.97±1.83	7.48±1.3	8.7±1.37	8.83±1.85	9.18±0.87
Lym%	73±5.92	71.28±4.8	69.58±4.72	70.8±6.84	73.03±4.96	71.31±4.14	71.3±2.41
Neu# (x10^9/L)	1.9±0.46	1.8±0.23	2.67±0.87	2.02±0.64	2.22±0.51	2.42±0.52	2.66±0.64
Neu%	19.07±5.19	18.77±3.61	20.93±3.89	19.09±5.49	17.74±3.56	18.04±3.65	18.84±1.86
Mon# (x10^9/L)	0.56±0.12	0.56±0.14	0.84±0.23	0.69±0.25	0.76±0.29	0.94±0.3	0.88±0.2
Mon%	5.62±1.43	5.77±0.74	6.69±1.4	6.46±1.8	6.13±2.23	6.77±0.3	6.21±0.35
Eos# (x10^9/L)	0.18±0.08	0.34±0.08	0.29±0.16	0.32±0.11	0.31±0.05	0.42±0.13 **	0.42±0.15 **
Eos%	1.73±0.36	3.22±0.53 **	2.23±0.65	3.01±0.89 *	2.49±0.39	3.14±0.95 **	2.99±0.72 *
Bas# (x10^9/L)	0.07±0.07	0.07±0.02	0.08±0.03	0.07±0.02	0.08±0.02	0.1±0.05	0.09±0.02
Bas%	0.6±0.27	0.7±0.2	0.59±0.18	0.67±0.14	0.63±0.12	0.73±0.24	0.66±0.19

A significant increase in hydrogen peroxide production by blood neutrophils was found in groups HCHD and HFHCHD, as compared to all the other groups ($p<0.001$), **(Figure 2)**.

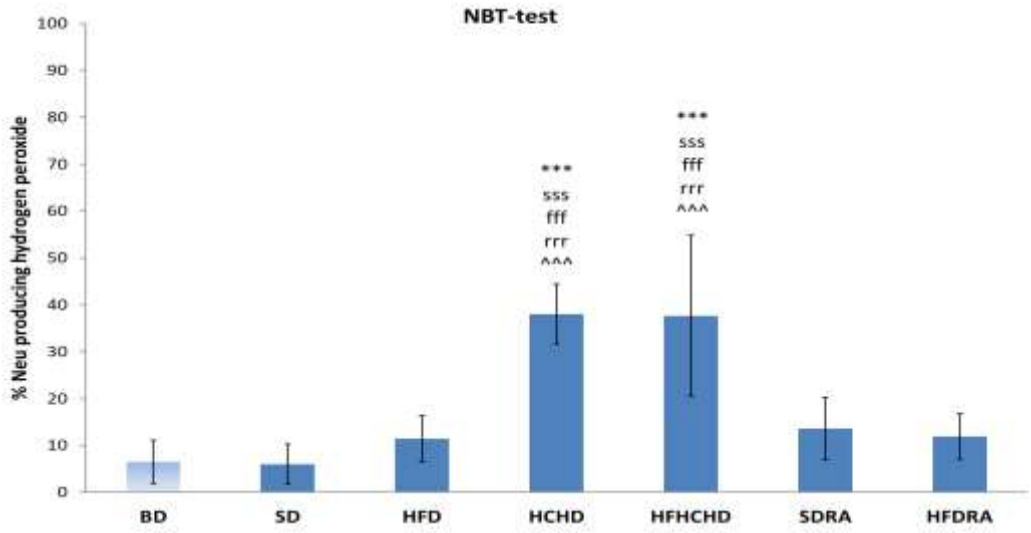

Figure 2. Percentage of neutrophils producing H_2O_2 in rats (n=8) before the start of the diet (BD) and in the groups SD (n=8), HFD (n=8), HCHD (n=8), HFHCHD (n=8), SDRA (n=8), HFDRA (n=8) after 5 weeks of application of the respective diets. Results are presented as mean values ± SD.
Statistically significant differences are indicated as follows: *** $p<0.001$ vs. BD; rrr $p<0.001$ vs. SDRA; sss $p<0.001$ vs. SD; fff $p<0.001$ vs. HFD; ^^^ $p<0.001$ vs. HFDRA.
Statistically significant differences are indicated as follows: * $p<0.05$ vs. BD; ** $p<0.01$ vs. BD.

DISCUSSION

A number of studies have established a relationship between metabolic disorders and changes in some hematological parameters, including counts of white blood cells (14). Some studies have even demonstrated that diet may have direct significant effect on some blood cells parameters (15). Although the mechanisms of such changes are not fully understood, research has proven that diet may change the lipid composition of the cell membrane (16). Our study has found no significant changes in total white blood cell counts and differential, except for eosinophils. Recent studies have revealed the protective role of eosinophils against obesity. In animals with high-fat diet induced obesity, reduced eosinophil numbers are associated with increased weight gain and impaired glucose tolerance. Conversely, an increase in eosinophils reduces high-fat diet induced obesity (17). Human studies do not support these claims, and some more recent animal studies also report conflicting results, so the relationship between eosinophils and obesity may not be unambiguous (10, 18). These divergent opinions could explain our results, in which no logical regularities could be found regarding eosinophils. In addition, some authors hypothesize that it is possible that there are different populations of eosinophils in adipose tissue with different physiological functions (18). Finally, our study has recorded the lowest eosinophil count before the beginning of the experimental diets, so the subsequent increase could be age-related (19).

Another interesting finding in our study is the increased production of hydrogen peroxide by blood neutrophils in some of the rats fed high-calorie diets. Over activation of neutrophils is often considered to be involved in the mechanism of oxidative stress development and inflammation. Oxidative stress is characterized as a state of imbalance between pro-oxidants and antioxidants, in which pro-oxidants predominate. High levels of reactive oxygen species have the potential to oxidize proteins and lipids, which subsequently leads to disturbances in energy metabolism, disruption of signaling pathways between and within cells, disruption of nutrient transport, and eventually cellular dysfunction and damage (20). Thus, oxidative stress is generally recognized as a major cause of aging processes and the development of various diseases and disorders – vascular disorders, insulin resistance, type 2 diabetes, metabolic syndrome, age-related degenerative diseases, etc. (21). Despite the presence of many enzymes and processes that generate oxide radicals, it should be emphasized that the majority of ROS (approximately 90%) are generated in the mitochondria as a result of

the functioning of the electron transport chain and the oxidative phosphorylation process (22). The data from our study demonstrated that in groups subjected to diets with high carbohydrate content (HCHD and HFHCHD), hydrogen peroxide production by blood neutrophils was significantly increased. It seems that increased sucrose intake stimulates either the NADPH-dependent pathway or the mitochondrial pathway of ROS generation. Although in the conditions of our experiment we cannot determine the exact mechanism of ROS generation, according to some researchers, the increased glucose concentration in patients with type 2 diabetes is the cause of increased production of hydrogen peroxide by neutrophils, which is probably involved in the mechanism of later tissue damage (23).

CONCLUSIONS

Diets with high content of sucrose have the potential to stimulate the production of hydrogen peroxide by blood neutrophils in experimental Wistar rats. Further study is needed to evaluate the changes in eosinophil count and function in response to high-calorie diets.

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Conflict of interests: The authors declare no conflict of interests.

REFERENCES

1. Lee, S. H., Park, S. Y. and Choi, C. S., Insulin resistance: from mechanisms to therapeutic strategies. *Diabetes & metabolism journal*, *46*(1): 15-37, 2022.
2. Aedh, A. I., Alshahrani, M. S., Huneif, M. A., Pryme, I. F. and Oruch, R., A glimpse into milestones of insulin resistance and an updated review of its management. *Nutrients*, *15*(4): 921, 2023.
3. Zhao, X., An, X., Yang, C., Sun, W., Ji, H. and Lian, F., The crucial role and mechanism of insulin resistance in metabolic disease. *Front Endocrinol (Lausanne), 14*: 1149239, 2023.
4. Salari-Moghaddam, A., Keshteli, A. H., Haghighatdoost, F., Esmaillzadeh, A. and Adibi, P., Dietary glycemic index and glycemic load in relation to general obesity and central adiposity among adults. *Clinical Nutrition*, *38*(6): 2936-2942, 2019.
5. Wang, L., Wang, H., Zhang, B., Popkin, B. M. and Du, S., Elevated fat intake increases body weight and the risk of overweight and obesity among Chinese adults: 1991–2015 trends. *Nutrients*, *12*(11): 3272, 2020.
6. Meex, R. C., Blaak, E. E. and van Loon, L. J., Lipotoxicity plays a key role in the development of both insulin resistance and muscle atrophy in patients with type 2 diabetes. *Obesity Reviews*, *20*(9): 1205-1217, 2019.
7. Bakinowska, E., Krompiewski, M., Boboryko, D., Kiełbowski, K. and Pawlik, A., The role of Inflammatory mediators in the pathogenesis of obesity. *Nutrients*, *16*(17): 2822, 2024.
8. De Luca, C. and Olefsky, J. M., Inflammation and insulin resistance. *FEBS letters*, *582*(1): 97-105, 2008.
9. Han, C. Y., Roles of reactive oxygen species on insulin resistance in adipose tissue. *Diabetes & metabolism journal*, *40*(4): 272, 2016.
10. Calco, G. N., Fryer, A. D. and Nie, Z., Unraveling the connection between eosinophils and obesity. *Journal of Leucocyte Biology*, *108*(1): 123-128, 2020.
11. Park, J. M., Lee, J. W., Shim, J. Y. and Lee, Y. J., Relationship between platelet count and insulin resistance in Korean adolescents: a nationwide population-based study. *Metabolic syndrome and related disorders*, *16*(9): 470-476, 2018.
12. Hulbert, A. J., Turner, N., Storlien, L. H. and Else, P. L., Dietary fats and membrane function: implications for metabolism and disease. *Biological Reviews*, *80*(1): 155-169, 2005.
13. Haga, Y., Ishii, K. and Suzuki, T., N-glycosylation is critical for the stability and intracellular trafficking of glucose transporter GLUT4. *Journal of biological chemistry*, *286*(36): 31320-31327, 2011.
14. Monteomo, G. F., Kamagate, A. and Yapo, A. P., Effects of metabolic syndrome on blood cells to Wistar rats. *J. Diabetes Metab. Disord. Control*, *5*(6): 222-225, 2018.
15. Souza, F. D. C. D. A., Duncan, W. P. and Carvalho, R. P., Hematology and plasma biochemistry in rats fed with diets enriched with fatty fishes from Amazon region. *Revista de Nutrição*, *27*: 547-555, 2014.

16. Unruh, D., Srinivasan, R., Benson, T., Haigh, S., Coyle, D., Batra, N., ... and Bogdanov, V. Y., Red blood cell dysfunction induced by high-fat diet: potential implications for obesity-related atherosclerosis. *Circulation*, *132*(20): 1898-1908, 2015.
17. De Oliveira, M. C., Silveira, A. L. M., de Oliveira, A. C. C., Lana, J. P., Costa, K. A., Vieira, É. L. M., ... and Ferreira, A. V. M., Eosinophils protect from metabolic alterations triggered by obesity. *Metabolism*, *146*: 155613, 2023.
18. Hu, Y. and Chakarov, S., Eosinophils in obesity and obesity-associated disorders. *Discovery Immunology*, *2*(1): kyad022, 2023.
19. Patel, S., Patel, S., Kotadiya, A., Patel, S., Shrimali, B., Joshi, N., ... and Jain, M., Age-related changes in hematological and biochemical profiles of Wistar rats. *Laboratory Animal Research*, *40*(1): 7, 2024.
20. Jiang, S., Liu, H. and Li, C., Dietary regulation of oxidative stress in chronic metabolic diseases. *Foods*, *10*(8): 1854, 2021.
21. Checa, J. and Aran, J. M., Reactive oxygen species: drivers of physiological and pathological processes. *Journal of Inflammation Research*, 1057-1073, 2020.
22. Turrens, J. F., Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, *552*(2): 335-344, 2003.
23. Pisarczyk-Wiza, D., Zozulinska-Ziolkiewicz, D., Wysocki, H. and Wierusz-Wysocka, B., Increase in glycaemia stimulates reactive oxygen species (ROS) production by polymorphonuclear neutrophils in type 2 diabetic patients. *Journal of Pre-Clinical and Clinical Research*, *5*(1), 2011.