E-ISSN 2667-5846

EXPERIMED

Volume **14** Issue **2 August** 2024

experimed.istanbul.edu.tr

ISTANBUL UNIVERSITY PRESS

EXPERIMED

INDEXING AND ABSTRACTING

Web of Science – Emerging Sources Citation Index
Scopus
ULAKBIM TR Index
Web of Science - Biological Abstracts
Web of Science - BIOSIS Previews
Chemical Abstracts Service (CAS)
EBSCO - Central & Eastern European Academic Source
SOBIAD

EXPERIMED

OWNER

Prof. Dr. Gunnur DENIZ

Department of Immunology, Istanbul University, Aziz Sancar Institute of Experimental Medicine, Istanbul, Turkiye

RESPONSIBLE MANAGER

Prof. Dr. Bedia CAKMAKOGLU

Department of Molecular Medicine, Istanbul University, Aziz Sancar Institute of Experimental Medicine, Istanbul, Turkiye

CORRESPONDENCE ADDRESS

Istanbul University, Aziz Sancar Institute of Experimental Medicine,
Vakıf Gureba Avenue, 34093, Capa, Fatih, Istanbul, Turkiye
Phone: +90 (212) 414 22 29
E-mail: experimed@istanbul.edu.tr

PUBLISHER

Istanbul Üniversitesi Yayınevi / Istanbul University Press
Istanbul University Central Campus,
34452 Beyazit, Fatih / Istanbul, Turkiye
Phone: +90 (212) 440 00 00

The publication language of the journal is English.

This is a scholarly, international, peer-reviewed and open-access journal published triannually in April, August and December.

Publication Type: Periodical

EXPERIMED

EDITORIAL MANAGEMENT BOARD

EXPERIMED

EDITORIAL BOARD

EXPERIMED

CONTENTS

Experimed 2024; 14(2): 63-67

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1427537

Exploring the Relationship Between HMGB1, CXCL12, CXCR4, and CXCR7 in the Context of Adriamycin-Induced Cardiotoxicity

Zeliha Emrence[1], Seyma Punar[2], Eylem Taskin[3], Celal Guven[4], Melda Sariman[5], Neslihan Abaci[1]

[1]Department of Genetics, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
[2]Department of Genetics, Institute of Health Sciences, Istanbul University, Istanbul, Turkiye,
[3]Department of Physiology, Faculty of Medicine, Adiyaman University, Adiyaman, Turkiye
[4]Department of Biophysics, Faculty of Medicine, Adiyaman University, Adiyaman, Turkiye
[5]Department of Genetics, Faculty of Dentistry, Istanbul Galata University, Istanbul Turkiye

ORCID ID: Z.E. 0000-0003-4809-6366; S.P. 0000-0002-7722-7401; E.T. 0000-0001-8172-4980; C.G. 0000-0003-0499-7787; M.S. 0000-0003-0898-529X; N.A. 0000-0002-9962-4010

Cite this article as: Emrence Z, Punar S, Taskin E, Guven C, Sariman M, Abaci N. Exploring the relationship between HMGB1, CXCL12, CXCR4, and CXCR7 in the context of adriamycin-induced cardiotoxicity. Experimed 2024; 14(2): 63-67.

ABSTRACT

Objective: High-mobility group box-1 (HMGB1), known as an abundant and highly conserved nuclear protein, plays a pivotal role in initiating inflammation, tissue healing, and the immune response following various forms of cell damage. The chemokine C-X-C motif chemokine ligand 12 (CXCL12) forms a signaling axis known as CXCL12/ CXCR4/CXCR7, along with the receptors CXCR4 and CXCR7. Our study aimed to explore the connection between HMGB1 and the involved chemokine axis, CXCR4, CXCL12, and CXCR7, in the context of adriamycin-induced cardiotoxicity.

Materials and Methods: We performed RNA interference to suppress HMGB1 expression in H9c2 cardiac myoblast cells. Adriamycin, an anti-tumor antibiotic known for causing cardiotoxicity, was used in conjunction with HMGB1 suppression. We investigated the combined and individual effects of these factors. Gene expression analysis was conducted through qRT-PCR 36 and 48 h post-treatment.

Results: Adriamycin treatment increased the expression of CXCL12, CXCR4, and CXCR7. Notably, our study observed significant changes in gene expression when HMGB1 was downregulated and adriamycin was administered. These findings suggest potential molecular mechanisms associated with adriamycin-induced cardiotoxicity, emphasizing the significance of the CXCR4/CXCL12 axis and the impact of HMGB1 modulation.

Conclusion: Our study provides insights into the molecular interplay between HMGB1 and the CXCL12/CXCR4/CXCR7 ligand-receptor axis in the context of adriamycin-induced cardiotoxicity. The results shed light on further research to enhance therapeutic approaches or advance new strategies to address this cardiotoxicity.

Keywords: Adriamycin, cardiotoxicity, CXCL12, CXCR4, CXCR7, gene expression, HMGB1

INTRODUCTION

Chemokines, a family of cytokines, play a pivotal role in regulating various cellular functions. They are primarily known for their essential roles in immune cell recruitment, which is crucial for the body's response to infections and tissue damage. By directing immune cells to specific sites within the body where their actions are most needed, chemokines influence various pathological conditions such as inflammation, atherosclerosis, hematopoiesis, and cancer (1).

The chemokine superfamily is composed of numerous chemokines and chemokine receptors. To date, more

Corresponding Author: Zeliha Emrence **E-mail:** zeliha.emrence@istanbul.edu.tr
Submitted: 30.01.2024 **Revision Requested:** 22.03.2024 **Last Revision Received:** 22.03.2024 **Accepted:** 28.03.2024 **Published Online:** 28.05.2024

than 50 chemokines and their receptors have been identified. Chemokines and their receptors are named using the term "chemokine" followed by an abbreviation for the ligand (L), and for chemokine receptors, the abbreviation (R) is used (2). The C-X-C motif chemokine ligand 12 (CXCL12) interacts with two seven-transmembrane domain G protein-coupled receptors: C-X-C chemokine receptor type 4 (CXCR4) and C-X-C chemokine receptor type 7 (CXCR7) (3). This interaction forms the CXCL12/CXCR4/CXCR7 axis, transmitting signals through its receptors; CXCR4 and CXCR7. High-mobility group box-1 (HMGB1), a highly conserved and abundant nuclear protein present in all eukaryotic cells, plays a key role in initiating inflammation, adaptive and innate immunity, as well as tissue healing following damage (4).

Unlike programmed apoptotic cell death, dead or dying cells release damage-associated molecular patterns (DAMPs). HMGB1 is the best-characterized DAMP, serving as a danger signal or alarm (5). Following cell death, HMGB1, a protein located inside the cell, is released (6). During stressful situations, HMGB1, a sensitive protein, is relocated from the nucleus to the secretory lysosomes in the cytoplasm or directly to the extracellular space. The released extracellular HMGB1 switches among different binding partners and receptors, triggering adaptive immunity and inflammation (5). Damaged tissue attracts immune cells, which then become active, and HMGB1 contributes to repair.

Post-transcriptional modifications in HMGB1 result in its functioning as different ligands for various receptors. For example, while acetylation doesn't appear to alter HMGB1's binding activity, it does induce changes in its redox state. Inside the cytoplasm and nucleus, HMGB1 has a strong reducing (redox) potential, but it becomes oxidized when it's outside the cell.

Upon reduction, HMGB1 forms a hetero-complex with the chemokine CXCL12, facilitating its subsequent binding to CXCR4. This interaction influences the balance of various signaling pathways, including G proteins and calcium pathways, as well as a range of signaling molecules such as beta-arrestins, JAK, GRK, MAPK, and PI3K kinases (5, 7).

Adriamycin (ADR), an antibiotic with anti-tumor properties, is used in the treatment of various types of cancer, including solid tumors, leukemia, and lymphomas (8). Also known as doxorubicin, adriamycin is an effective drug that targets cancer cells to inhibit their growth and proliferation (9). However, this treatment can lead to cardiotoxicity, in addition to side effects such as nausea, vomiting, hair loss, and hematopoietic suppression (10). Symptoms can vary, ranging from arrhythmia to heart failure, during or after the treatment process (11). Luo et al. showed that HMGB1 contributes to ADR induced cardiotoxicity by upregulating autophagy (10).

In a previous study, we demonstrated that the inhibition of HMGB1 could prevent cardiac cell loss by suppressing the apoptotic pathway (12). The severe cardiotoxicity of ADR limits its clinical applications, and its mechanisms are not yet fully understood. Our study aimed to explore the relationship between HMGB1 and the receptors CXCR4 and CXCR7, in conjunction with the chemokine CXCL12, within the H9c2 cell line. The goal of this investigation was to shed light on the mechanisms underlying cardiotoxicity following adriamycin treatment.

MATERIALS AND METHODS

Cell Culture

The H9c2 cell line comprising cardiac myoblast cells was obtained from ATCC and cultured using DMEM as per ATCC recommendations. Cells were sub-cultured every 2–3 days upon reaching 70%–80% confluence.

Knockdown of HMGB1 by RNA Interference and Adriamycin Treatment

To test our hypothesis, we created four main experimental groups using exponentially growing H9c2 cardiac myoblast cells: (I) Control group treated with non-targeting small interfering RNA (siRNA); (II) siRNA group treated with 10 nM HMGB1 siRNA; (III) ADR group treated with 2 µM adriamycin, and (IV) siRNA + ADR group treated with both siRNA and adriamycin. Cells were transfected with HMGB1 siRNA (GE Healthcare Dharmacon, Lafayette, CO, United States) for knockdown (13), using non-targeting siRNA (Healthcare Dharmacon, Lafayette, CO, United States) as a negative control. Transfection was performed using a HiPerFect Transfection Reagent (Qiagen, Valencia, CA). We tested various ratios of siRNA and transfection reagent at different time points for transfection optimization. Based on these trials, we decided to use a 10 nM siRNA dose and 3 µL of transfection reagent (for a 24-well plate). Due to the ineffectiveness of achieving a significant knockdown within 24 h, we chose incubation times of 36 and 48 h for the experiments. siRNA and ADR applications were conducted simultaneously, and each group was replicated.

Expression Analysis

The real time–polymerase chain reaction (RT-PCR) technique was utilized to explore the effects of administered siRNA on gene expression and to determine the rate of gene knockdown in the cells. The PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts) was used to isolate total RNA, adhering to the instructions provided by the manufacturer. The isolated RNA was then employed for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts). The reverse transcription process was executed using random hexamers, following the manufacturer's instructions under suitable conditions. The synthesized cDNAs and primers, with sequences provided separately, were used to perform RT-PCR. The LightCycler 480 Instrument II device (Roche Diagnostic, Mannheim, Germany) and SYBR detection method were used for expression analysis.

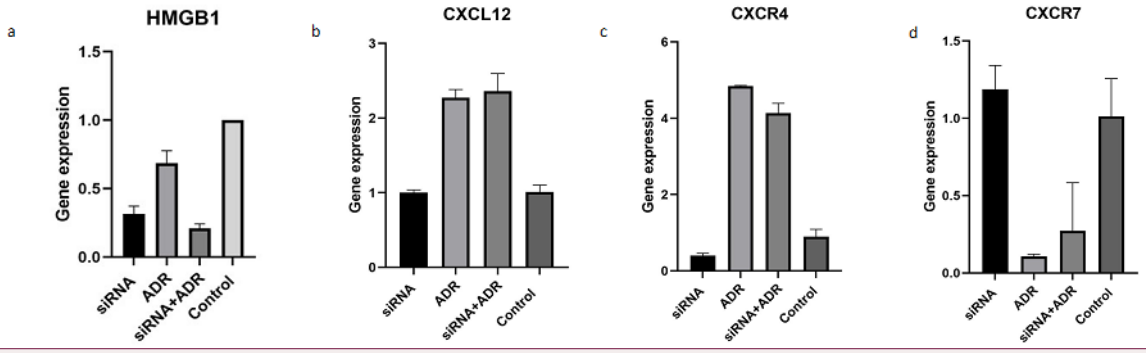

Figure 1. Expression of HMGB1 (a), CXCL12 (b), CXCR4 (c), and CXCR7 (d) in H9c2 cells after transfection of HMGB1 siRNA and/or ADR treatment. The expression levels of genes were detected 36 h after transfection by quantitative PCR.
siRNA: cells were transfected with HMGB1 siRNA; ADR: cells were treated with adriamycin; HMGB1; siRNA + ADR: cells were transfected with HMGB1 siRNA and treated with ADR; Control: untreated cells.

Each sample was replicated, and the mean value was computed. The $2^{-(\Delta\Delta ct)}$ method (14) was employed for gene expression analysis. The expression levels of the genes were normalized to those of the housekeeping gene, beta-actin. The primers were designed based on the *Rattus norvegicus* reference genome, Rnor_6.0.

B-actin F: 5′-TCTGAACCCTAAGGCCAACCGTG-3′

B-actin R: 5′-AACACAGCCTGGATGGCTACGT-3′

HMGB1 F: 5′-AAACATGGGCAAAGGAGATCC-3′

HMGB1 R: 5′-AGTTGACAGAAGCATCCGGGT-3′

CXCL12 F: 5′-CCAGAGCCAACGTCAAACAT-3′

CXCL12 R: 5′-GTTGTTGCTTTTCAGCCTTGC-3′

CXCR4 F: 5′-GTTGTTGCTTTTCAGCCTTGC-3′

CXCR4 R: 5′-AAGAGTGTCCACCCCGTTTC-3′

CXCR7 F: 5′-TTGCTGTCCCCTTCACCATC-3′

CXCR7 R: 5′-CAAAATGGTACGGCAGCCAA-3′

Statistical Analyses

All data analyses were conducted using the Statistical Package for Social Sciences (SPSS version 21.0, SPSS Inc., Chicago, IL, USA). To determine if there was a statistically significant difference in gene expression levels, we employed the Kolmogorov-Smirnov test. $p<0.05$ was considered statistically significant for determining significance.

RESULTS

Gene Expression Changes in 36 Hours

We investigated the role of ADR in chemokines through the HMBG1 gene. The knockdown level of HMGB1 was determined using RT-PCR. A decrease in HMGB1 expression by 69% was observed 36 h post-siRNA transfection in H9c2 cells (Figure 1a). The expression of the chemokine ligand CXCL12 was not significantly changed in HMGB1 knockdown cells. However, it increased by 2.3-fold in cells treated with ADR. In cells treated with both ADR and siRNA, the increase was slightly higher at 2.35-fold ($p<0.05$; Figure 1b).

The expression of the chemokine receptor CXCR4 was decreased by 0.61-fold, compared to the control in HMGB1 knockdown cells. Conversely, it was increased by 4.8-fold in cells treated with ADR. In cells treated with both ADR and siRNA, the Increase was slightly lower at 4.1-fold ($p<0.05$; Figure 1c).

In HMGB1 knockdown cells, there was no significant change in the expression of the CXCR7 receptor. However, in cells treated with ADR and both ADR and siRNA, similar decreases of 0.9-fold and 0.7-fold, respectively, were observed ($p<0.05$; Figure 1d).

Gene Expression Changes in 48 Hours

The expression of HMGB1 was downregulated by 72% 48 h after siRNA transfection in H9c2 cells (Figure 2a). The expression of the CXCL12 ligand was increased by 2.7-fold in cells treated with ADR. In cells treated with a combination of ADR and siRNA, the increase was slightly higher at 31-fold ($p<0.05$; Figure 2b). The expression of the CXCR4 receptor had a significant increase in ADR-treated cells, with a 53-fold increase. In cells treated with both ADR and siRNA, the increase was slightly lower at 31-fold ($p<0.05$; Figure 2c). The expression of the CXCR7 receptor was decreased by 0.91-fold in ADR-treated cells and by 0.93-fold in cells treated with both ADR and siRNA ($p<0.05$; Figure 2d).

DISCUSSION

Adriamycin, a chemotherapy drug used in the treatment of leukemia, neuroblastoma, breast cancer, lymphoma, Ewing's syndrome, and osteosarcoma, disrupts DNA repair by intercalating into DNA via topoisomerase II, generating free radicals that cause DNA and protein damage. However, its clinical use can induce cardiac toxicity by triggering an

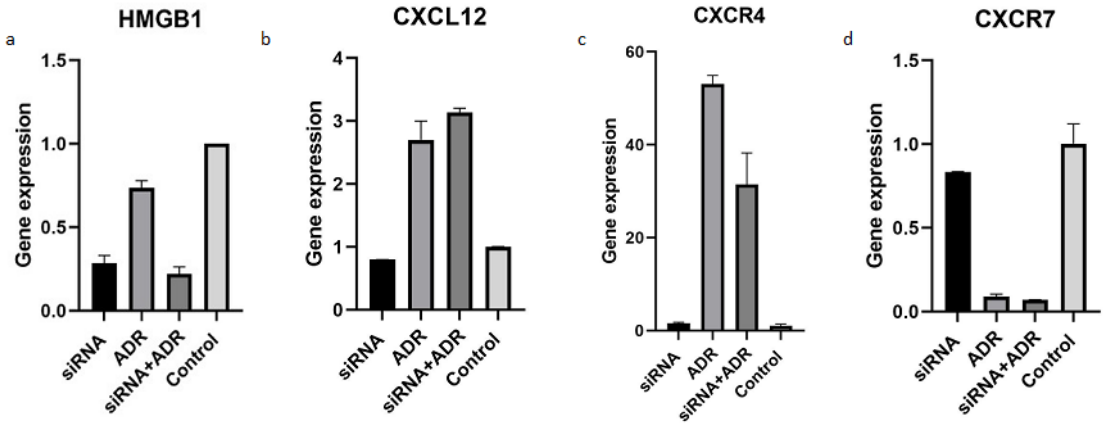

Figure 2. Expression of HMGB1 (a), CXCL12 (b), CXCR4 (c), and CXCR7 (d) in H9c2 cells after transfection of HMGB1 siRNA and/or adriamycin (ADR) treatment. The expression levels of genes were detected 48 h after transfection by quantitative PCR.
siRNA: cells were transfected with HMGB1 siRNA; ADR: cells were treated with adriamycin; HMGB1; siRNA + ADR: cells were transfected with HMGB1 siRNA and treated with ADR; Control: untreated cells.

inflammatory response through immune cells, thereby increasing the risk of cardiac failure (15).

When tissues are damaged, endogenous molecules known as DAMPs are released, with HMGB1 being one of these DAMPs associated with cellular damage (5). Innate immune cells recognize DAMPs through specific pattern recognition receptors (PRRs), including Toll-like receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, and C-type lectin receptors. Activation of these receptors upon sensing DAMPs initiates inflammatory pathways, leading to the release of chemokines (15).

Inflammatory mediators and chemokines release signal for neutrophils to detach from circulation and migrate to the site of damage. Neutrophils, constituting the majority of granulocytes in peripheral blood (50%–70%), play a vital role in the innate immune system's defense mechanisms.The process of neutrophil formation, known as granulopoiesis, involves maturation stages regulated by the cytokine G-CSF, disrupting the CXCR4-CXCL12 interaction and assisting in retaining neutrophils in the bone marrow. G-CSF induces the release of circulating neutrophils by reducing CXCL12 expression in the bone marrow and decreasing CXCR4 on neutrophils (16). This mechanism disrupts the retention of neutrophils in the bone marrow, leading to their release into circulation.

Our study contributes to understanding the CXCR4-CXCL12 relationship and its relevance to neutrophil release (16), thereby impacting the inflammatory process and their recruitment to sites of tissue damage. This understanding holds significance for various pathological conditions characterized by inflammation and tissue damage, such as cancer, autoimmune diseases, and infections.

Our study aimed to down-regulate HMGB1 and examine its relationship with the chemokine CXCL12, as well as the CXCL12 receptors CXCR4 and CXCR7. We also sought to understand the effects of the chemotherapy ADR. We analyzed the expression of these genes in rat myoblast cells treated with ADR alone and in combination with HMGB1 siRNA. Interestingly, we observed no significant change in HMGB1 gene expression in cells treated solely with ADR. This finding aligns with Luo et al.'s demonstration that ADR treatment increased HMGB1 release and that HMGB1 silencing could potentially reverse ADR-induced cardiac toxicity. However, since our analysis was conducted at the RNA level, we did not observe an increase in HMGB1 expression (10).

In our examination of CXCL12 expression in cells treated with HMGB1 siRNA, we found that the down-regulation of HMGB1 at 36 and 48 h did not lead to a significant change. However, the application of ADR increased CXCL12 expression in both 36 and 48h samples. When we compared samples treated with both siRNA and ADR to those treated with only ADR, we observed that HMGB1 suppression did not significantly affect CXCL12 expression. This finding is consistent with a study by Beji and colleagues on human cardiac mesenchymal progenitor cells, which showed that ADR application increased the expression of this chemokine (17).

In our study, we observed an increase in the expression of the chemokine receptor CXCR4 in cells treated with ADR. However, in cells subjected to a combined treatment of siRNA and ADR, the expression of CXCR4 decreased compared to cells treated solely with ADR. This decrease in CXCR4 expression may be attributed to the reduction in heterocomplex formation that occurs when HMGB1 is downregulated. A study conducted by Schiraldi et al. demonstrated that the recruitment of inflammatory cells induced by HMGB1 depends on CXCL12. HMGB1 and CXCL12 form a heterocomplex that exerts its

effects through the CXCR4 receptor. This study revealed that the HMGB1-CXCL12 heterocomplex induces. distinct conformational re-arrangements of CXCR4 compared to CXCL12 alone (7). When examining the expression of the CXCR7 receptor, we observed a significant decrease in expression in samples treated solely with ADR and in samples treated with a combination of siRNA and ADR, compared to the control.

In our study, we found that the knockdown of HMGB1 did not cause any change in the expression of CXCR7, while it did affect the expression of CXCR4. CXCR7 plays a role in decreasing CXCL12 levels in mammalian cells and signals independently. Through various mechanisms, CXCR7 modulates the expression and activity of CXCR4 and CXCL12. Interestingly, CXCL12 initially has a higher affinity for CXCR7 than CXCR4. However, the binding and dissociation rates of CXCL12 for CXCR7 are slower than those for CXCR4, favoring kinetic binding to CXCR4 (18).

Our study demonstrated that the expression of CXCR4 changes depending on HMGB1 expression. These results suggest that in the context of ADR-induced cardiotoxicity, the expression of CXCL12 occurs through the CXCL12/CXCR4 axis. In conclusion, ADR treatment reveals new potential molecular mechanisms associated with cardiotoxicity. This finding opens the possibility for further research to enhance existing therapeutic approaches or develop new strategies for managing cardiotoxicity.

Ethics Committee Approval: Ethics committee approval is not required due to the use of commercial cell lines.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Z.E., E.T., C.G, N.A; Consultant: Z.E.; Experiments: Z.E., S.P., M.S.; Drafting Manuscript- Z.E., N.A.; Critical Revision of Manuscript- N.A.; Final Approval and Accountability- Z.E., E.T., C.G, N.A., S.P., M.S.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) as a Project No: 114S118.

REFERENCES

1. Shi Y, Riese DJ, Shen J. The role of the CXCL12/CXCR4/CXCR7 chemokine axis in cancer. Front Pharmacol 2020; 11: 574667.
2. Huynh C, Dingemanse J, Meyer zu Schwabedissen HE, Sidharta PN. Relevance of the CXCR4/CXCR7-CXCL12 axis and its effect in pathophysiological conditions. Pharmacol Res 2020; 161: 105092.
3. Segret A, Rücker-Martin C, Pavoine C, Flavigny J, Deroubaix E, Châtel MA, et al. Structural localization and expression of CXCL12 and CXCR4 in rat heart and isolated cardiac myocytes. J Histochem Cytochem 2007; 55(2): 141-50.
4. De Leo F, Quilici G, Tirone M, De Marchis F, Mannella V, Zucchelli C, et al. Diflunisal targets the HMGB 1/ CXCL 12 heterocomplex and blocks immune cell recruitment. EMBO Rep 2019; 20(10): 47788.
5. Bianchi ME, Crippa MP, Manfredi AA, Mezzapelle R, Rovere Querini P, Venereau E. High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. Immunol Rev 2017; 280(1): 74–82.
6. Gardella S, Andrei C, Ferrera D, Lotti L V, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep 2002; 3(10): 995-1001.
7. Schiraldi M, Raucci A, Muñoz LM, Livoti E, Celona B, Venereau E, et al. HMGB1 promotes recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and signaling via CXCR4. J Exp Med 2012; 209(3): 551-63.
8. Mordente A, Meucci E, Martorana GE, Giardina B, Minotti G. Human heart cytosolic reductases and anthracycline cardiotoxicity. IUBMB Life 2001; 52(1-2): 83-8.
9. Gao Y, Shang Q, Li W, Guo W, Stojadinovic A, Mannion C, et al. Antibiotics for cancer treatment: A double-edged sword. J Cancer 2020; 11(17): 5135-49.
10. Luo P, Zhu Y, Chen M, Yan H, Yang B, Yang X, et al. HMGB1 contributes to adriamycin-induced cardiotoxicity via up-regulating autophagy. Toxicol Lett 2018; 292:115–22.
11. Mitry MA, Edwards JG. Doxorubicin induced heart failure: Phenotype and molecular mechanisms. Int J Cardiol Heart Vasc 2016; 10: 17-24
12. Taskin E, Guven C, Kaya ST, Sariman M, Emrence Z, Sirma Ekmekci S, et al. Silencing HMGB1 expression inhibits adriamycin's heart toxicity via TLR4 dependent manner through MAPK signal transduction. JBUON 2020; 25(1): 554–65.
13. Qin WD, Mi SH, Li C, Wang GX, Zhang JN, Wang H, et al. Low shear stress induced HMGB1 translocation and release via PECAM-1/PARP-1 pathway to induce inflammation response. PLoS One 2015; 10(3): 0120586
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25(4): 402–8.
15. Bhagat A, Shrestha P, Kleinerman ES. The innate immune system in cardiovascular diseases and its role in doxorubicin-induced cardiotoxicity. Int J Mol Sci 2022; 23(23): 14649.
16. Rosales C. Neutrophil: A cell with many roles in inflammation or several cell types? Front Physiol 2018; 9: 113.
17. Beji S, Milano G, Scopece A, Cicchillitti L, Cencioni C, Picozza M, et al. Doxorubicin upregulates CXCR4 via miR-200c/ZEB1-dependent mechanism in human cardiac mesenchymal progenitor cells. Cell Death Dis 2017; 8(8): 3020.
18. Mezzapelle R, Leo M, Caprioglio F, Colley LS, Lamarca A, Sabatino L, et al. CXCR4/CXCL12 activities in the tumor microenvironment and implications for tumor immunotherapy. Cancers (Basel) 2022; 14(9): 2314.

Experimed 2024; 14(2): 68-72

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1443398

The Effects of *Panax ginseng* on Serum Oxidative Stress Following Bisphenol A Exposure

Najiullah Fazalyar[1], Seren Ede Pazarbasi[1,2], Dogancan Dorucu[3], Goksel Sener[4], Tugba Tunali-Akbay[5]

[1]Department of Biochemistry, Institute of Health Sciences, Marmara University, Istanbul, Turkiye
[2]Vocational School of Health Sciences, Fenerbahce University, Istanbul, Turkiye
[3]Department of Urology, Faculty of Medicine, Marmara University, Istanbul, Turkiye
[4]Department of Pharmacology, Faculty of Pharmacy, Fenerbahçe University, Istanbul, Turkiye
[5]Department of Basic Medical Sciences, Faculty of Dentistry, Marmara University, Istanbul, Turkiye

ORCID ID: N.F. 0009-0009-0725-8702; S.E.P. 0000-0002-3195-4064; D.D. 0000-0002-9714-3336; G.S. 0000-0001-7444-6193; T.T.A. 0000-0002-2091-9298

Cite this article as: Fazalyar N, Ede Pazarbasi S, Dorucu D, Sener G, Tunali-Akbay T. The effects of *Panax ginseng* on serum oxidative stress following bisphenol A exposure. Experimed 2024; 14(2): 68-72.

ABSTRACT

Objective: Bisphenol A (BPA) is a toxic compound that causes oxidative stress by disrupting antioxidant enzymes and promoting tissue lipid peroxidation. This study aimed to examine the impacts of BPA on serum oxidative stress in rats and to detect the antioxidant feature of *Panax ginseng* (PxG) in reducing BPA-induced oxidative stress.

Materials and Methods: Wistar Albino rats (250-300 g) were divided into control, control + PxG, BPA, and BPA + PxG groups. 50 mg/kg BPA and 100 mg/g PxG were given for six weeks. Serum total antioxidant and oxidant status, lipid peroxidation, and glutathione levels were determined.

Results: BPA administration increased total oxidant status and lipid peroxidation, while PxG administration to the BPA group decreased these parameters. PxG also increased total antioxidant status and glutathione levels compared to the BPA group.

Conclusion: BPA was seen to cause an increase in oxidative parameters and PxG administration to restore the oxidative stress that was generated after BPA exposure, suggesting that this may help to prevent the adverse effects caused by BPA exposure.

Keywords: Bisphenol A, *Panax ginseng*, oxidative stress, antioxidant effect

INTRODUCTION

Bisphenol A (BPA), is an industrial product produced in most plastic manufacturing industries (1), and causes oxidative stress by suppressing antioxidant levels and increasing lipid peroxidation and free radicals (2). BPA is a toxic monomer that damages DNA through an oxidative process (3). A relationship exists between BPA consumption and a high risk of kidney impairment and liver damage (4). BPA-associated oxidative stress leads to the disruption of cellular homeostasis, which causes inflammation and tissue damage responses (5). Apart from the liver and kidneys, BPA has been linked to a number of other health problems due to oxidative stress, such as heart-related diseases (4), and metabolic disorders (1), which suggests the far-reaching nature of this chemical regarding all aspects of human health.

Because research on the properties of traditional plants has revealed many bioactive compounds to reduce oxidative

Corresponding Author: Tuğba Tunalı-Akbay **E-mail:** ttunali@marmara.edu.tr
Submitted: 27.02.2024 **Revision Requested:** 14.03.2024 **Last Revision Received:** 06.04.2024 **Accepted:** 03.05.2024 **Published Online:** 31.05.2024

damage or strengthen the antioxidant system, the tendency toward natural compound research has increased. *Pistacia integerrima* (6), grape seed (7), sweet potato (8), Tualang honey (9), and sesame lignans (10) have been demonstrated to have preventive benefits regarding tissue damage caused after BPA exposure. The present study investigated the possible effects of *Panax ginseng* (PxG) on BPA-induced oxidative stress.

PxG, or Korean ginseng, is an enduring plant from the Araliaceae family (11) and grows mainly in East Asia and North America (12). It contains several bioactive molecules including ginsenosides, polyphenols, and phytosterol, which play a significant role in certain pharmacological effects (13, 14). Ginsenosides have potent antioxidant effects and can inhibit oxidative damage. Ginsenosides' reactive oxygen species scavenging properties are responsible for their protective action (13). PxG has been shown to have a regulating effect on blood glucose levels, offering protection from cardiovascular risk factors (14), as well as to improve cognitive function, physical tolerance (15), and sexual function (16).

An *in vitro* investigation has shown PxG to act as an anti-inflammatory on BPA-treated A549 lung cells by decreasing reactive oxygen species generation and affecting NF-κB activation and COX-2 expression (17). Ok et al. found ginseng to increase the anti-apoptotic mechanisms that inhibit BPA-induced apoptosis (18). Research on the influence of PxG administration regarding BPA-related abnormalities in the uterus and liver of ovariectomized mice has indicated PxG to protect these mice from chemotaxis generated by BPA and inflammatory reactions (19).

MATERIALS AND METHODS

Experimental Animal Model

Marmara University Animal Care and Use Ethics Committee approved the experimental stages of this study (Protocol Number: 57. 2023mar). The study used 32 rats (Wistar Albino, male), weighing an average of 250-300 grams. Conventional conditions were used throughout this stage. The rats were separated into Control (C), C + PxG, BPA, and BPA + PxG groups, with eight rats in each group. BPA was purchased from Sigma-Aldrich (Germany) and dissolved in 1% ethanol. PxG was obtained from Casel İlaç Sanayi A.Ş. (Turkiye) and dissolved in water.

The C group of rats were under a standard diet (BPA vehicle, orally). The C + PxG group was administered PxG (100 mg/kg, orally with BPA vehicle). The BPA group was administered 50 mg/kg BPA (orally). The BPA + PxG group was given BPA (50 mg/kg, orally, and 100 mg/kg of PxG, orally). BPA and PxG were administered to the rats for six weeks, five days per week. Trunk blood was used to prepare the serum samples.

Determining Serum Lipid Peroxidation and Glutathione (GSH)

For determining the lipid peroxidation, thiobarbituric acid makes a colorful complex with malondialdehyde (MDA) (20). The absorbance of the pink color resulting from the MDA-thiobarbituric acid reaction was measured by spectrophotometry. The GSH level was determined using the Beutler test (21), which involves reduction of Ellman's reagent with SH groups to generate yellow-colored 5, 5'-dithiobis (2-nitrobenzoic acid).

Determining the Serum Total Oxidant Status (TOS) and Antioxidant Status (TAS)

TOS and TAS were determined spectrophotometrically (RL No: 0017, RL No: 0024, respectively, Rel Assay Diagnostics, Gaziantep, Turkiye). The research used the antioxidant method to determine the ability of serum to counteract free-radical reactions and the oxidant measurement method to measure the ability of serum oxidants to change a ferrous ion to a ferric ion.

Oxidative Stress Index (OSI) Calculation

The following formula was utilized to calculate the OSI:

OSI = TOS (μmol H_2O_2 eq/L) / TAS (μmol Trolox eq/L)

Table 1: Serum TAS, TOS, and OSI Levels

	C	BPA	C+PxG	BPA+PxG	p-value
TAS (μmol/L)‡	1.78 ± 0.04	1.55 ± 0.09*,+■	2.31 ± 0.11*	2.25 ± 0.08*,+	0.0001
TOS (μmol/L)‡	5.80 ± 0.22	10.95 ± 0.39*,+■	4.37 ± 0.30*	4.72 ± 0.29*,+	0.0001
OSI‡	0.034 ± 0.002	0.075 ± 0.005*,+■	0.019 ± 0.002*	0.021 ± 0.003*,+	0.0001

C: control group; BPA: BPA exposed group; C + PxG: control + Panax ginseng group; BPA + PxG: BPA exposed + Panax ginseng administered group; TAS: total antioxidant status; TOS = total oxidant status; OSI = oxidative stress index

*Compared to the C group;

+Compared to the BPA group;

■ Compared to the BPA + PxG group;

‡: Mean ± SD

Statistical Analyses

GraphPad Software (GraphPad Prism 9.0, California, USA) was used to conduct the statistical analysis, with means and standard deviations (SD) being used to present the data. Analysis of variance (ANOVA) and the post hoc Tukey test were used to compare the groups, with a $p < 0.05$ being accepted as significant.

RESULTS

TAS, TOS, and OSI Levels

BPA administration decreased TAS and increased TOS and OSI significantly, compared to the C group (Table 1). PxG administration significantly increased total antioxidant status, significantly decreased TOS, and reduced OSI, compared to the C group (Table 1). When compared to the BPA group, the BPA + PxG group showed a significant increase in total antioxidant status and a significant reduction in TOS and OSI.

MDA and GSH Levels

According to Figure 1, administering BPA to the C group increased MDA levels significantly, and administering PxG to the BPA group decreased their MDA levels. In addition, MDA levels in the BPA + PxG group increased significantly compared to the C + PxG group (Figure 1). Applying BPA to the rats did not change their GSH levels (Figure 1). Administering PxG to the C group and BPA groups significantly increased the GSH levels in both groups (Figure 1).

DISCUSSION

The widespread use of BPA and its potential health consequences are still being researched. This study has determined the possible effects of BPA on health, especially its potential effects on serum oxidative stress, and also determined the role of PxG, primarily its antioxidant properties, on these effects.

The excessive generation of free radicals causes peroxidative alterations, which eventually result in increased lipid peroxidation, which could be used used as a diagnostic indicator of tissue damage (22). Many studies have highlighted how BPA damages tissues through oxidative stress (23, 24). Ge et al. found BPA to restrict the growth of Sertoli cells by causing oxidative stress, whereas low-dose BPA promotes cell growth by stimulating the metabolism with regard to energy (25). Tiwari et al. found that, although BPA is not carcinogenic, it does have genotoxic effects (26). Similar to these studies, administering BPA to rats in the current study increased their serum lipid peroxidation levels and oxidative stress. Compared to the results presented by Ozaydin et al. and Moghaddam et al., which revealed a decrease in GSH levels in BPA-exposed groups (24, 27), this study detected no significant changes in serum GSH levels after BPA exposure. The lack of change in serum GSH levels following BPA administration indicates that the body may metabolize BPA through different detoxification pathways. As a traditional medicine that has been used for centuries, PxG is an Asian plant with an antioxidant potential due to its ginsenoid content. PxG could assist in the 'body's

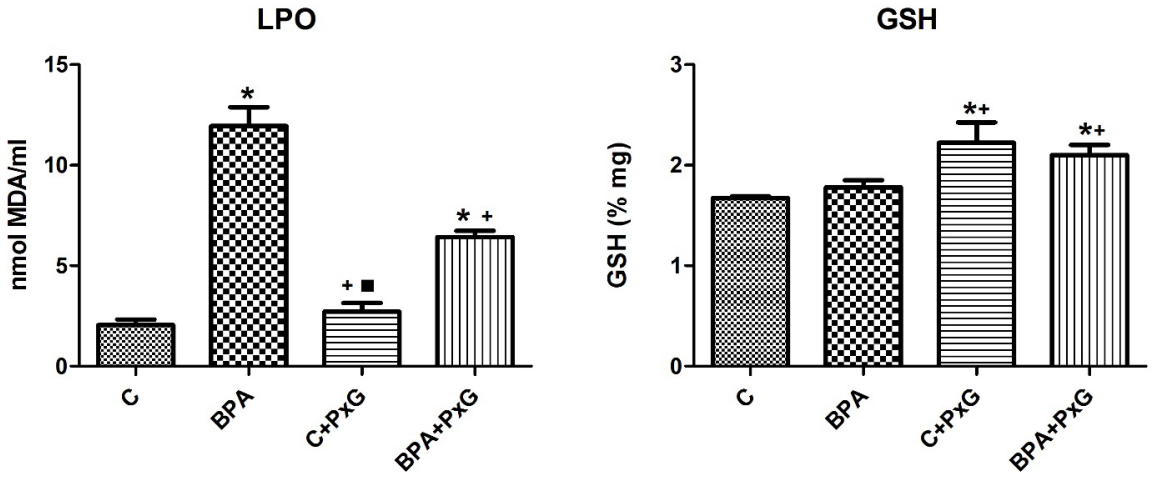

Figure 1. Serum MDA and GSH levels.
C: control group; BPA: BPA exposure group; C + PxG: control + Panax ginseng group; BPA + PxG: BPA exposure + Panax ginseng administered group; MDA: malondialdehyde; LPO: lipid peroxidation; GSH: glutathione.
* Compared to the C group;
+ Compared to the BPA group;
■ Compared to the BPA + PxG group

neutralization of free radicals, thus preventing cells from oxidative damage. Kim et al. showed PxG to reduce serum lipid peroxidation and to increase antioxidant enzyme activities after exercise-induced oxidative stress (28). Ramesh et al. have shown PxG to ameliorate age-related oxidative damage in tissues (29). Chung et al. demonstrated PxG to significantly increase the total antioxidant capacity in postmenopausal women (30). Consistent with these studies, the current study has shown PxG to increase serum TAS and GSH levels in rats exposed to BPA, while decreasing TOS and lipid peroxidation. This highlights the possible anti-oxidative capacity of PxG for reducing BPA-induced oxidative stress. The study has also showed administering PxG to healthy rats to also increase TAS and GSH levels, suggesting potential protective effects of PxG against oxidative stress.

In conclusion, PxG can decrease BPA-induced oxidative damage and may potentially be an efficient protector against other environmental contaminants. Because of its antioxidant properties, PxG may prove useful in treating oxidative stress-related diseases.

Ethics Committee Approval: Marmara University Animal Care and Use Ethics Committee approved the experimental stages of this study (Protocol Number: 57. 2023mar).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- T.T.A., G.S.; Data Acquisition- N.F., S.E.P., D.D.; Data Analysis/Interpretation- T.T.A.; Drafting Manuscript- N.F., T.T.A.; Critical Revision of Manuscript- T.T.A.; Final Approval and Accountability- T.T.A., G.S.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was funded by a grant from the Marmara University Scientific Research Projects Unit (TYL-2024-11206)

REFERENCES

1. Rubin BS. Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. J Steroid Biochem Mol Biol 2011; 127(1-2): 27-34.
2. Gassman NR. Induction of oxidative stress by bisphenol A and its pleiotropic effects. Environ Mol Mutagen 2017; 58(2): 60-71.
3. Xin F, Jiang L, Liu X, Geng C, Wang W, Zhong L, et al. Bisphenol A induces oxidative stress-associated DNA damage in INS-1 cells. Mutat Res Genet Toxicol Environ Mutagen 2014; 769: 29-33.
4. Erler C, Novak J. Bisphenol a exposure: human risk and health policy. J Pediatr Nurs 2010; 25(5): 400-7.
5. Meli R, Monnolo A, Annunziata C, Pirozzi C, Ferrante MC. Oxidative stress and BPA toxicity: an antioxidant approach for male and female reproductive dysfunction. Antioxidants 2020; 9(5): 405.
6. Ishtiaq A, Bakhtiar A, Silas E, Saeed J, Ajmal S, Mushtaq I, et al. Pistacia integerrima alleviated Bisphenol A induced toxicity through Ubc13/p53 signalling. Mol Biol Rep 2020; 47: 6545-59.
7. Abdou HM, Abd Elkader HAE, El-Gendy AH, Eweda SM. Neurotoxicity and neuroinflammatory effects of bisphenol A in male rats: The neuroprotective role of grape seed proanthocyanidins. Environ Sci Pollut Res Int 2022; 29(6): 9257-68.
8. Johnson M, Pace RD. Sweet potato leaves: properties and synergistic interactions that promote health and prevent disease. Nutr Rev 2010; 68(10): 604-15.
9. Zaid SS, Othman S, Kassim NM. Potential protective effect of Tualang honey on BPA-induced ovarian toxicity in prepubertal rat. BMC Complement Altern Med 2014; 14: 509.
10. El Bana E, Kamal K, Shaheen N, Abowafa S. The protective role of sesame oil against bisphenol A-induced cardiotoxicity: A histological and immunohistochemical study. Kasr Al Ainy Med J 2019; 25(3): 87-98.
11. Yun TK. Brief introduction of Panax ginseng C.A. Meyer. J Korean Med Sci 2001; 16 Suppl(Suppl): S3-5.
12. Potenza MA, Montagnani M, Santacroce L, Charitos IA, Bottalico L. Ancient herbal therapy: A brief history of Panax ginseng. J Ginseng Res 2023; 47(3): 359-65.
13. He B, Chen D, Zhang X, Yang R, Yang Y, Chen P, et al. Oxidative stress and ginsenosides: An update on the molecular mechanisms. Oxid Med Cell Longev 2022; 2022: 9299574.
14. Liu H, Lu X, Hu Y, Fan X. Chemical constituents of Panax ginseng and Panax notoginseng explain why they differ in therapeutic efficacy. Pharmacol Res 2020; 161: 105263.
15. Coleman CI, Hebert JH, Reddy P. The effects of Panax ginseng on quality of life. J Clin Pharm Ther 2003; 28(1): 5-15.
16. Leung KW, Wong AS. Ginseng and male reproductive function. Spermatogenesis 2013; 3(3): e26391.
17. Song H, Lee YY, Park J, Lee Y. Korean Red Ginseng suppresses bisphenol A-induced expression of cyclooxygenase-2 and cellular migration of A549 human lung cancer cell through inhibition of reactive oxygen species. J Ginseng Res 2021; 45(1): 119-25.
18. Ok S, Kang JS, Kim KM. Cultivated wild ginseng extracts upregulate the anti-apoptosis systems in cells and mice induced by bisphenol A. Mol Cell Toxicol 2017; 13(1): 73-82.
19. Lee J, Park J, Lee YY, Lee Y. Comparative transcriptome analysis of the protective effects of Korean Red Ginseng against the influence of bisphenol A in the liver and uterus of ovariectomized mice. J Ginseng Res 2020; 44(3): 519-26.
20. Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma. Methods Mol Biol 1998; 108: 101-6.
21. Beutler E. Red cell metabolism: a manual of biochemical methods. 3rd ed.; Grune&Stratton; Orlando; FL, 1984.
22. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014; 2014: 360438.
23. Zhang H, Yang R, Shi W, Zhou X, Sun S. The association between bisphenol A exposure and oxidative damage in rats/mice: A systematic review and meta-analysis. Environ Pollut 2022; 292(Pt B): 118444.
24. Ozaydin T, Oznurlu Y, Sur E, Celik I, Uluisik D, Dayan MO. Effects of bisphenol A on antioxidant system and lipid profile in rats. Biotech Histochem 2018; 93(4): 231-8.
25. Ge LC, Chen ZJ, Liu H, Zhang KS, Su Q, Ma XY, et al. Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: a comparative proteomic analysis. Biochim Biophys Acta 2014; 1840(9): 2663-73.
26. Tiwari D, Kamble J, Chilgunde S, Patil P, Maru G, Kawle D, et al. Clastogenic and mutagenic effects of bisphenol A: an endocrine disruptor. Mutat Res 2012; 743(1-2): 83-90.

27. Moghaddam HS, Samarghandian S, Farkhondeh T. Effect of bisphenol A on blood glucose, lipid profile and oxidative stress indices in adult male mice. Toxicol Mech Methods 2015; 25(7): 507-13.
28. Kim SH, Park KS, Chang MJ, Sung JH. Effects of Panax ginseng extract on exercise-induced oxidative stress. J Sports Med Phys Fitness 2005; 45(2): 178-82.
29. Ramesh T, Kim SW, Sung JH, Hwang SY, Sohn SH, Yoo SK, et al. Effect of fermented Panax ginseng extract (GINST) on oxidative stress and antioxidant activities in major organs of aged rats. Exp Gerontol 2012; 47(1): 77-84.
30. Chung TH, Kim JH, Seol SY, Kim YJ, Lee YJ. The Effects of Korean red ginseng on biological aging and antioxidant capacity in postmenopausal women: a double-blind randomized controlled study. Nutrients 2021; 13(9): 3090.

Experimed 2024; 14(2): 73-84

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1384602

Gene Expression Profile as a Precursor of Inflammation in Mouse Models: BFMI860 and C57BL/6NCrl

Ayca Dogan[1], Gudrun A. Brockmann[2]

[1]Department of Physiology, Faculty of Medicine, Altinbas University, Istanbul, Turkiye
[2]Animal Breeding Biology and Molecular Genetics, Albrecht Daniel Thaer-Institute for Agricultural and Horticultural Sciences, Humboldt University of Berlin, Berlin, Germany

ORCID ID: A.D. 0000-0002-6020-8327; G.A.B. 0000-0002-4387-2947

Cite this article as: Dogan A, Brockmann GA. Gene expression profile as a precursor of inflammation in mouse models: BFMI860 and C57BL/6NCrl. Experimed 2024; 14(2): 73-84.

ABSTRACT

Objective: We aimed to investigate the differences in the immune response to body fat content between the genetically mutant obese BFMI860 (BFMI) mouse strain and the lean C57BL/6Ncrl (B6) mouse strain as a control and the effects of obesity on gene expression on inflammation-related pathways in epididymal adipose tissue.

Materials and Methods: Six males from each strain were maintained on a standard maintenance diet (SMD) or a high-fat diet (HFD). At the age of 10 weeks, serum and epididymal adipose tissue samples were collected for cytokine and gene expression analyses. RNA samples from epididymal adipose tissue were hybridized using the microarray technique to study the quantitative transcript amounts of genes.

Results: Pathway analysis of gene expression data revealed no considerable development of inflammatory state in BFMI and B6 on SMD. Both strains responded to HFD distinctly; the inflammatory state was more prominent in the obese BFMI group than in the lean B6 group. Several genes, such as *Adipoq*, *NFkbia, Plaur, F2r, C3ar1*, and *Nfatc4* in pathways involved in the immune system have been found to be differentially regulated in BFMI mice. Under the condition of obesity in BFMI mice, the induction of inflammation-related pathways indicates an increased risk of insulin resistance, atherosclerosis, and cardiovascular disease.

Conclusion: This study identified distinct expression patterns of genes involved in inflammatory pathways, particularly those associated with the adipocytokine signaling pathway and complement and coagulation cascades, in the epididymal adipose tissue of BFMI and B6 mice. The BFMI strain is a valuable and promising model for clarifying the mechanisms underlying obesity and the activation of inflammation in adipose tissue.

Keywords: Obesity, high-fat diet, adipocytokine signaling pathway, complement and coagulation cascade

INTRODUCTION

The comorbidities associated with obesity are due to the infiltration and accumulation of pro-inflammatory macrophages in the adipose tissue, which trigger changes in the immune system through the production of inflammatory cytokines. Positive correlations between inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein 1 (MCP1), interleukin-6 (IL-6), and interleukin-8 (IL-8), and the degree of adiposity in obese individuals have been presented through several studies (1). Increased circulating levels of inflammatory markers are associated with obesity-related pathologies. The distribution of adipose tissue affects these associations. Visceral adipose tissue is a major risk factor for insulin resistance, type 2 diabetes, cardiovascular disease, and metabolic syndrome (1). Therefore, visceral fat is considered as the most dangerous adipose tissue (2). It is known that obesity causes alterations in the transcript levels of genes in some pathways linking fat tissue metabolism to

Corresponding Author: Ayca Dogan **E-mail:** ayca.mollaoglu@altinbas.edu.tr
Submitted: 01.11.2023 **Revision Requested:** 03.03.2024 **Last Revision Received:** 22.06.2024 **Accepted:** 05.08.2024

the immune system (2). Therefore, to investigate the effects of obesity on the immune response, we examined the transcript levels of cytokines and pro-inflammatory genes involved in inflammation-related pathways including adipocytokine signaling pathway and complement and coagulation cascades, in the Berlin Fat Mouse Inbred 860 (BFMI) strain and the C57BL/6NCrl (B6) strain. BFMI is genetically predisposed to obesity and characterized by high body fat mass, impaired fat oxidation (3), dysregulated cytokine production, and insulin resistance (4). The B6 strain is a lean inbred strain often used as a reference.

The objectives of the present study were, first, to detect differences in the immune response to body fat content between BFMI and B6 mice at the genetic level (strain effect) and, second, to investigate the effects of obesity on gene expression levels in inflammation-related pathways within each strain.

MATERIALS AND METHODS

Animals and Ethical Approval

BFMI and B6 (Charles River Laboratories, Sulzfeld, Germany) male mice were bred, fed, and grown until 10 weeks old in an animal facility at Humboldt University of Berlin, as described previously (3). In brief, we generated the BFMI860 strain from an outbred population. Founder mice were originally purchased from pet shops in Berlin and subsequently selected for low protein content, low body mass and high fat content, followed by high fatness for 58 generations before inbreeding. C57BL/6NCrl mice (Charles River Laboratories, Sulzfeld, Germany) were used as controls. All experiments were performed in accordance with the approval of the German Animal Welfare Authorities (approval no. G0152/04 and V54-19c20/15c MR 17/1).

Animal Feeding

BFMI (n=6) and B6 (n=6) mice were fed either with a standard maintaining diet (SMD) or high-fat diet (HFD), for 7 weeks. Following weaning at 3 weeks old, 6 male B6 mice were fed with SMD (V1534-000 ssniff R/M-H, ssniff Spezialdiäten GmbH, Soest, Germany) containing 12.8 MJ/kg metabolizable energy (9% of its energy from fat, 33% from protein content and 58% from carbohydrates). Additionally, 6 male BFMI mice were given an HFD (S8074-E010 ssniff EF R/M, ssniff Spezialdiäten GmbH, Soest, Germany) containing 19.1 MJ/kg metabolizable energy, with 45% of its energy from fat, 24% from protein, and 31% from carbohydrates. The SMD included soy oil as its source of fat, whereas the HFD included coconut oil and suet as its source of fat. The mice had *ad libitum* access to food and water. They were maintained at room temperature (22°C - 24°C) with a 12-hour light/dark cycle. The detailed composition and energy content of both diets were extensively documented by Wagener et al. (3). Mice were weighed weekly from the age of 3 weeks.

Intraperitoneal Glucose Tolerance Test (IPGTT)

After 12-14 h of fasting, we collected a baseline blood sample (fasting glucose at time zero) from 10-week-old mice. Each animal was given a single intraperitoneal injection of glucose (B. Braun, Melsungen, Germany) at a dose of 2 g/kg body weight. Blood was drawn from the tail tip 15, 30, 60, and 120 min after intraperitoneal glucose injection. Glucose concentrations

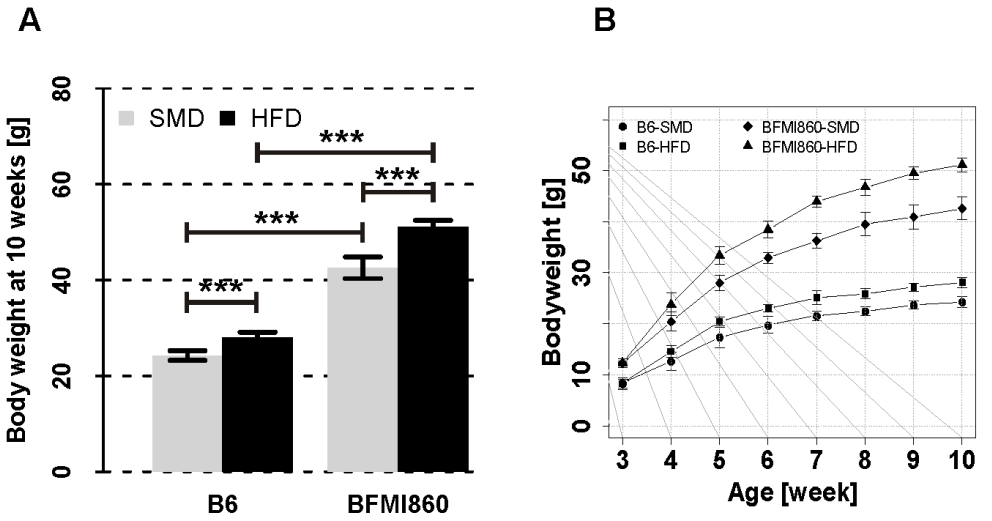

Figure 1A. Body weight at 10 weeks of age, **B.** Body weight development of BFMI and B6 males under either SMD or HFD from 3 to 10 weeks of age. Each point represents the mean weight and standard deviation (SD). Statistically significance difference; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

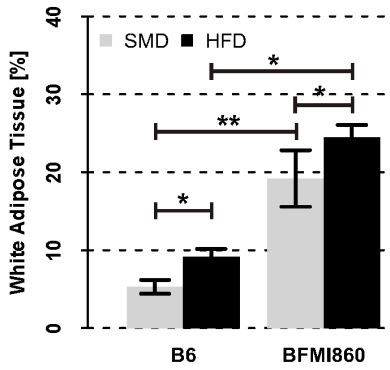

Figure 2. Percentage of white adipose tissue (WAT) to body weight of BFMI and B6 males under either SMD or HFD at 10 weeks of age. Values are mean ± SD. Statistically significance difference; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

were measured using an Ascensia Elite glucose analyzer (Bayer HealthCare, Leverkusen, Germany). For the determination of insulin levels, blood samples were drawn from the tail and extracted at 0, 30, 60, and 120 min after intraperitoneal injection of glucose and were determined by enzyme-linked immunosorbent assay (ELISA) as described.

Animal Euthanasia and Sample Collection

Ten-week-old male mice were anesthetized with isoflurane and decapitated after 2 hours of fasting period. Blood was collected at necropsy after cervical dislocation. Serum was recovered by centrifugation for 15 min at 600 g and used for cytokine measurements, and epididymal adipose tissue was used for gene expression analyses. Tissues were collected in liquid nitrogen and stored at -80°C until RNA preparation.

Measurement of Serum Parameters

Serum leptin, adiponectin, and insulin levels were measured by ELISA as described by Hantschel et al. (4). Leptin levels

Table 1. Differentially expressed genes in the adipocytokine signaling pathway in the epididymal adipose tissue.

MGI ID	Symbol	Gene name	BFMI vs B6 SMD			BFMI vs B6 HFD		
			Fold	FDR	p-value	Fold	FDR	p-value
99484	*Chuk*	conserved helix-loop-helix ubiquitous kinase	0.821	0.258	NS	0.739	0.002	<0.001
104741	*Nfkbia*	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	0.947	NS	NS	4.094	0.000	<0.001
1342774	*Ppargc1a*	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	0.707	0.004	<0.001	0.735	0.036	NS
97374	*Npy*	neuropeptide Y	1.678	0.007	<0.001	1.592	0.000	<0.001
104740	*Ppara*	peroxisome proliferators activated receptor alpha	0.868	0.094	NS	0.737	0.000	<0.001
98214	*Rxra*	retinoid X receptor alpha	0.689	0.001	<0.001	0.614	0.000	<0.001
106675	*Adipoq*	adiponectin, C1Q and collagen domain containing	0.706	0.263	NS	0.458	0.001	<0.001
93830	*Adipor2*	adiponectin receptor 2	0.640	0.013	<0.001	0.593	0.040	NS
95755	*Slc2a1*	solute carrier family 2 (facilitated glucose transporter), member 1	1.105	NS	NS	2.108	0.023	<0.001
1201791	*Socs3*	suppressor of cytokine signaling 3	1.173	NS	NS	1.841	0.000	<0.001
98216	*Rxrg*	retinoid X receptor gamma	0.837	0.114	NS	0.595	0.000	<0.001

MGI, Mouse Genome Informatics; FDR, false discovery rates; SMD, standard maintenance diet; HFD, high-fat diet; BFMI, BFMI860 mouse strain; B6, C57BL/6Ncrl mouse strain; NS: not significant

The table shows all genes that were significantly differentially expressed at $p<0.001$ and $FDR<0.5$ in at least one of the comparisons between BFMI and B6 on SMD or BFMI and B6 on HFD. MGI ID refers to the gene identity number in Mouse Genome Informatics database (www.informatics.jax.org/genes.shtml).

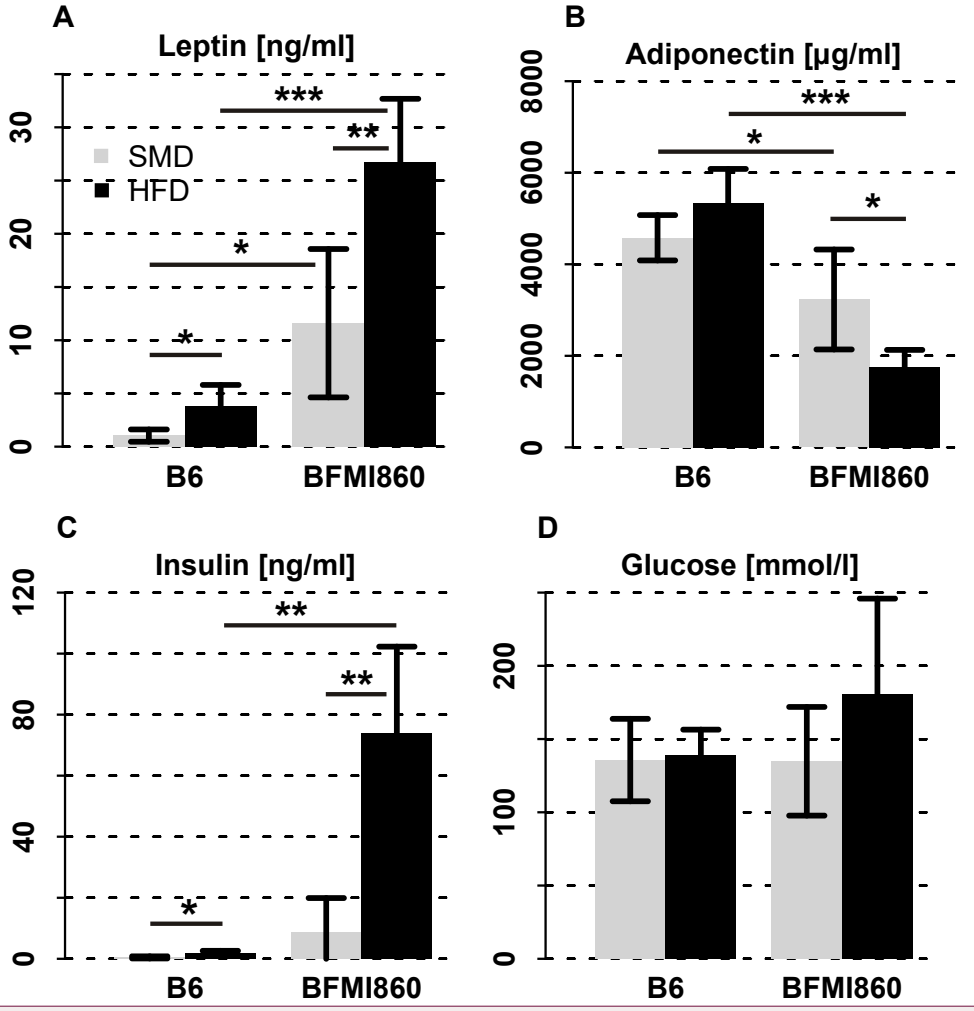

Figure 3. Serum levels of **A**. leptin, **B.** adiponectin, **C.** insulin, **D.** glucose at 10 weeks of age. Data are expressed as mean ± SD (n= 6). Statistically significance difference; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

were determined using an m/rLeptin ELISA kit (Mediagnost, Reutlingen, Germany). Adiponectin levels were determined using a DuoSet ELISA Development kit (R&D Systems, Wiesbaden, Germany). Insulin levels were measured using a commercial Insulin Mouse Ultrasensitive ELISA (DRG Instruments GmbH, Marburg, Germany).

Gene Expression Analysis

Gene expression was measured using RNA isolated from epididymal adipose tissue of BFMI and B6 mice at 10 weeks. RNA was extracted and gene expression was performed as described in Wagener et al. (3). The RNA samples of six animals of the four biological groups (B6-SMD / B6-HFD / BFMI-SMD / BFMI-HFD) were hybridized on Illumina Mouse WG-6 v1.1 Expression BeadChips using an Illumina BeadStation 500X.

The lists of differentially expressed genes in epididymal adipose tissue are illustrated in Table 1 for adipocytokine signaling pathway, and in Table 2 for the complement and coagulation cascades. Diet effects on the adipocytokine signaling pathway and complement and coagulation cascades are shown in Tables 3 and 4, respectively. Differentially expressed genes in epididymal adipose tissue, in the interaction between strain and diet, and immune response-related pathways are noted in Tables 5 and 6.

Statistical Analyses

Fluorescence images of the Illumina Bead Arrays were translated into relative expression levels using the Bioconductor (5) package bead array (6) with standard parameter settings. The intensity values of the arrays were log2-transformed and quantile-normalized for fat tissue. The statistical analysis was performed using the R statistical software package (7). Differences in gene expression between BFMI and B6 mice fed with either a SMD or a HFD and between HFD and SMD in either BFMI or B6 mice were evaluated using two separate Student's t-test. To address multiple testing, false discovery rates (FDR) were calculated using a customized algorithm that was implemented in R. Where necessary, medians of fold-

Table 2. Differentially expressed genes in complement and coagulation cascade signaling pathway in the epididymal adipose tissue.

MGI ID	Symbol	Gene name	BFMI vs B6 SMD			BFMI vs B6 HFD		
			Fold	FDR	p-value	Fold	FDR	p-value
97611	*Plau*	plasminogen activator, urokinase	1.822	0.009	<0.001	2.101	0.004	<0.001
97612	*Plaur*	plasminogen activator, urokinase receptor	1.613	0.048	NS	1.965	0.010	<0.001
101802	*F2r*	coagulation factor II (thrombin) receptor	1.583	0.039	NS	2.438	0.001	<0.001
88381	*F3*	coagulation factor III	0.473	0.012	<0.001	1.354	0.131	NS
109325	*F7*	coagulation factor VII	2.971	0.001	<0.001	1.994	0.000	<0.001
103107	*F10*	coagulation factor X	2.404	0.020	<0.001	2.380	0.000	<0.001
105975	*H2-Bf*	histocompatibility 2 complement component	1.319	0.052	NS	2.576	0.006	<0.001
87931	*Adn*	complement factor D (adipsin)	0.320	0.014	<0.001	0.055	0.000	<0.001
88226	*C2*	complement component 2	0.201	0.000	<0.001	0.290	0.000	<0.001
105937	*Cfi*	complement component factor i	1.266	0.019	<0.001	1.577	0.002	<0.001
1097680	*C3ar1*	complement component 3a receptor 1	2.463	0.011	<0.001	4.237	0.000	<0.001

MGI ID, Mouse Genome Informatics ; FDR, false discovery rates; SMD, standard maintenance diet; HFD, high-fat diet; BFMI, BFMI860 mouse strain; B6, C57BL/6Ncrl mouse strain; NS, not significant.
The table shows all genes that were significantly differentially expressed at p<0.001 and FDR<0.5 in at least one of the comparisons between BFMI and B6 on SMD or BFMI and B6 on HFD. MGI ID refers to the gene identity number in Mouse Genome Informatics database (www.informatics.jax.org/genes.shtml).

Table 3. Differentially expressed genes in the adipocytokine signaling pathway in the epididymal adipose tissue.

MGI ID	Symbol	Gene name	B6 HFD vs SMD			BFMI HFD vs SMD		
			Fold	FDR	p-value	Fold	FDR	p-value
107899	*Cd36*	Cd36 antigen	0.5	0.002	<0.001	1.2	0.874	NS
104741	*Nfkbia*	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	0.4	0.000	<0.001	1.6	0.297	NS
106675	*Adipoq*	adiponectin, C1Q and collagen domain containing	0.8	0.028	<0.001	0.5	0.131	NS

MGI, Mouse Genome Informatics; FDR, false discovery rates; SMD, standard maintenance diet; HFD, high-fat diet; BFMI, BFMI860 mouse strain; B6, C57BL/6Ncrl mouse strain; NS, not significant.
The table shows all genes that were significantly differentially expressed at p<0.001 and FDR<0.5 in at least one of the comparisons between BFMI and B6 on SMD or BFMI and B6 on HFD. MGI ID refers to the gene identity number in the Mouse Genome Informatics (MGI) database (www.informatics.jax.org/genes.shtml).

Table 4. Differentially expressed genes in complement and coagulation cascade signaling pathway in the epididymal adipose tissue.

MGI ID	Symbol	Gene name	B6 HFD vs SMD			BFMI HFD vs SMD		
			Fold	FDR	p-value	Fold	FDR	p-value
88381	*F3*	coagulation factor III	0.5	0.020	<0.001	1.3	0.205	NS
98736	*Thbd*	thrombomodulin	0.4	0.011	<0.001	0.6	0.366	NS
105975	*H2-Bf*	histocompatibility 2 complement component	0.5	0.036	<0.001	0.9	0.658	NS
87931	*Adn*	complement factor D (adipsin)	1.0	0.779	NS	0.2	0.035	<0.001

MGI, Mouse Genome Informatics ; FDR, false discovery rates; SMD, standard maintenance diet; HFD, high-fat diet; BFMI, BFMI860 mouse strain; B6, C57BL/6Ncrl mouse strain; NS, not significant.

The table shows all genes that were significantly differentially expressed at p<0.001 and FDR<0.5 in at least one of the comparisons between BFMI and B6 on SMD or BFMI and B6 on HFD. MGI ID refers to the gene identity number in Mouse Genome Informatics (MGI) database (www.informatics.jax.org/genes.shtml).

Table 5. Differentially expressed genes show interaction between strain and diet in the epididymal adipose tissue.

KEGG Pathway	MGI ID	Symbol	Gene Name	Fold
Adipocytokine signaling pathway	1194882	*Irs3*	insulin receptor substrate 3	0.4
	107899	*Cd36*	CD36 antigen	2.5
	98216	*Rxrg*	retinoid X receptor gamma	0.7
Adipocytokine signaling, T cell signaling, B cell signaling, and Toll-like receptor signaling pathway	1099800	*Nfkb2*	nuclear factor of kappa light polypeptide gene enhancer in B-cells	1.6
	104741	*Nfkbia*	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	4.3
T cell signaling	88332	*Cd3e*	CD3 antigen, epsilon polypeptide	1.6
T cell signaling, Natural killer cell signaling	1920431	*Nfatc4*	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	1.9
T cell signaling, Natural killer cell signaling, Fc epsilon RI signalling pathway	1342293	*Lat*	linker for activation of T cells	2.3
B cell signaling	1096398	*Cd81*	CD81 antigen	1.4
Natural killer cell signaling	95904	*H2-K1*	histocompatibility 2, K1, K region	3.5
	1346866	*Map2k1*	mitogen activated protein kinase 1	2.5
Complement and coagulation cascades	88381	*F3*	coagulation factor III	2.9
	87931	*Adn*	complement factor D (adipsin)	0.2

KEGG, Kyoto Encyclopedia of Genes and Genomes; MGI, Mouse Genome Informatics.

The table shows all genes that were significantly differentially expressed at p<0.001 and FDR<0.5 in at least one of the comparisons between BFMI and B6 on SMD or BFMI and B6 on HFD. MGI ID refers to the gene identity number in the Mouse Genome Informatics (MGI) database (www.informatics.jax.org/genes.shtml).

Table 6. Gene annotations of differentially expressed genes in the epididymal adipose tissue.

BFMI vs B6 on HFD fat	Number of genes	Genes
Immune system Toll-like receptor signaling pathway	18	*Cxcl9, Irf5, Ly96, Ifna5, Fos, Tlr5, Tlr2, Spp1, Cd14, Tlr1, Tlr7, Myd88, Ccl4, Ccl3, Ticam2, Pik3cg, Nfkbia, Map2k1*
Leukocyte transendothelial migration	8	*Atp1b3, Rap1b, Vav1, Bcar1, Plcg2, Ncf2, Pik3cg, Myl9*
B cell receptor signaling pathway	14	*Cd72, Ptpn6, Sykb, Prkcb, Card11, Blnk, Kras, Fos, Vav1, Lilrb3, Btk, Plcg2, Pik3cg, Nfkbia*
Natural killer cell mediated cytotoxicity	19	*H2-K1, Cd48, Itgb2, Ptpn6, Fcgr4, Kras, Vav1, Plcg2, Grb2, Pik3cg, Sykb, Prkcb, Sh3bp2, Fcgr3, Ifna5, Tyrobp, Fcer1g, Lat, Map2k1*
Signaling molecules and interaction		
Cytokine-cytokine receptor interaction	27	*Vegfa, Ccl6, Cxcl9, Ccl24, Tnfsf13, Ccr5, Ccl2, Ccl4, Figf, Ccl3, Pf4, Il10, Ccl27a, Ccl17, Tspan7, Cxcl1, Ltbr, Ifna5, Tnfrsf17, Cxcl14, Ccl7, Cx3cr1, Pdk3, Ctf1, Il10rb, Ccl8, Bmp2,*
BFMI vs B6 on SMD		
Immune system		
Leukocyte transendothelial migration	17	*Rock2, , Cldn1, Ncf4, Esam, Actn1, Ctnna1, Ncf1, Gnai2, Cyba, Rapgef3, F11r, Cldn5, Gnai1, Mmp2, Mmp9, Rac2, Cxcr4,*
Antigen processing and presentation 5		*Ctss, H2-Ea, Ifi30, Ctsb, Cd74*
Signaling molecules and interaction		
ECM-receptor interaction	11	*Col1a2, Col6a1, Itga6, Col6a2, Col1a1, Col3a1, Itga5, Vtn, Col4a1, Spp1, Lamb3*
Signal transduction		
Wnt signaling pathway	16	*Smad2, Rock2, Lrp6, Prkcb, Sfrp2, Rac3, Wnt2b, Wif1, Sfrp4, Smad4, Wnt2, Cxxc4, Sox17, Siah1a, Rac2, Fzd6*
HFD vs SMD in BFMI		
PPAR signaling pathway	5	*Scd1, Fabp3, Hmgcs2, Scd2, Ucp1*

MGI, Mouse Genome Informatics ; FDR, false discovery rates; SMD, standard maintenance diet; HFD, high-fat diet; BFMI, BFMI860 mouse strain; B6, C57BL/6Ncrl mouse strain.
Down-regulated genes are underlined. Only annotations with corrected $p \leq 0.001$ are shown. MGI ID refers to the gene identity number in the Mouse Genome Informatics (MGI) database (www.informatics.jax.org/genes.shtml).

changes, medians of significance values and FDR were used to define unique values for each gene, if multiple oligos existed and were measuring the expression of the same gene.

To measure the significance of the interaction between strain and diet, a 2 by 2 analysis of variance (ANOVA) was performed with the factors strain and diet for each probe within fat tissue:

$\log_2(\text{expression}) = \mu + \text{strain} + \text{diet} + \text{strain: diet} + \epsilon$

where μ is the probe mean, strain is either BFMI860 or B6, diet is either SMD or HFD, strain: diet is the interaction term and ϵ is the residual variance. The R/Bioconductor package maanova (8) was used with the maanova functions, read. madata, fitmaanova, and matest.

Differentially expressed genes with p values <0.001 and FDRs <0.5 were considered statistically significant in ANOVA and Student's t-test.

Genes showing significant differences between groups were assigned to inflammatory-related pathways based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Pathways were visualized using the software GenMAPP (9).

RESULTS

Weight Gain and Serum Parameters

At 10 weeks, BFMI males were nearly two times as heavy as B6 males (Figure 1A). BFMI mice were already heavier at the early age of 3 weeks (Figure 1B). Body weight gain was markedly higher in the HFD group than in the SMD group in both strains. In addition, BFMI mice stored proportionally more fat than B6 in response to HFD (Figure 2).

Serum concentrations of leptin, adiponectin, insulin, and glucose were compared between BFMI and B6 control mice fed SMD and HFD. As expected, serum leptin levels were higher in BFMI mice than in B6 mice on both diets due to the higher body fat mass in BFMI (Figure 3A). Serum adiponectin levels were increased in B6 mice and decreased in BFMI mice on HFD (Figure 3B). Therefore, the changes in adiponectin levels in response to different diets were strain-dependent. BFMI mice on HFD exhibited high blood glucose levels. No significant change in blood glucose levels was observed in B6 mice (Figure 3D). In addition, serum insulin levels were markedly increased in response to HFD in BFMI mice but not in B6 mice (Figure 3C).

Gene Expression Analysis

We initially investigated the adipocytokine signaling pathway to identify differences in the regulation of gene expression that cause subsequent metabolic system alterations. Then, we examined the complement and coagulation cascades that were significantly altered in epididymal fat tissue. Subsequently, we studied the B-cell receptor, T-cell receptor, toll-like receptor, and natural killer cell signaling pathways to explore the interaction between strain and diet in epididymal fat tissue. Genes were considered differentially expressed if $p<0.001$ and FDR<0.5 within affected pathways.

The differentially expressed genes in epididymal adipose tissue belonging to different pathways are presented in Tables 1 and 2.

Strain Effect

Adipocytokine Signaling Pathway

KEGG analysis revealed that the adipocytokine signaling pathway was differentially expressed between BFMI and B6 mice. Two and seven of the 67 genes in this pathway were differentially expressed between these strains on SMD and HFD, respectively (Table 1). The expressions of Ppargc1a and Adipor2 were significantly down-regulated in BFMI vs. B6 mice fed with SMD. In the case of HFD, we detected decreased *Adipoq, Chuk, Ppara,* and *Rxrg* expressions and markedly increased *Nfkbia, Socs3*, and *Slc2a1* expressions in BFMI vs. B6 mice. We also observed higher leptin and lower *Irs1* transcript levels in the BFMI group, although these alterations were not statistically significant.

Complement and Coagulation Cascades

Group analysis of the differential gene expression pattern provided evidence that the complement and coagulation cascades were up-regulated in BFMI vs. B6 mice. Five out of 63 genes were up-regulated, while three were down-regulated in BFMI vs. B6 mice on SMD; eight genes were up-regulated and two were down-regulated on HFD, respectively. Among these genes, transcript levels of *H2-Bf* increased and *F3* decreased dissimilarly in BFMI vs. B6 mice on HFD and SMD, respectively. On *HFD, Plaur* and *F2r* were up-regulated (Table 2). We detected increased transcript levels in *Plaur, F7, F10, Cfi,* and *C3ar1* and decreased expression in *Adn* and *C2* of BFMI vs. B6 in both feeding groups.

Diet Effect

Adipocytokine Signaling Pathway

We found that expression of *Irs3, Cd36, Nfkbia*, and *Adipoq* was significantly reduced in HFD vs SMD in B6 mice (Table 3). However, no marked alterations were determined in the gene expression of BFMI mice.

Complement and Coagulation Cascades

Decreased expressions of *F3, thrombomodulin* (*Thbd*), and *H2-Bf* genes were observed in B6 mice in HFD vs. SMD (Table 4). We detected decreased *Adn* expression, which was only differentially expressed in BFMI mice.

Effect of Interaction Between Strain and Diet

KEGG analysis identified T-cell receptor and natural killer cell signaling pathways that were significantly up-regulated in the epididymal adipose tissue with respect to strain and diet. The genes *Nfatc4* and Lat, which contribute to both pathways, were differentially expressed. In the T-cell, B-cell, and toll-like receptor signaling pathways, *Nfkb2* and *Nfkbia* were differentially expressed. The expressions of all four genes were significantly increased in (BFMI on HFD vs. SMD) vs. (B6 on HFD vs. SMD), indicating the effects of interaction between strain and diet. We also detected decreased *Irs3* and increased *Cd36*, *Cd3e*, and *Cd81* expression in BFMI mice (Table 5).

Data Mining

To detect differentially expressed genes that did not belong to any of the analyzed pathways, genes were annotated using GeneCodis. The differentially expressed genes were then assigned to related pathways. Due to the large dataset size, several pathways that could affect the immune response were selected to provide an overview of inflammation. The most dramatically modulated genes, which are not involved in the discussed pathways, were observed in BFMI vs. B6 mice on HFD. The complete list of differentially expressed genes (up- and down-regulated) is shown in Table 6.

Analyzing the diet effects (HFD vs SMD), the genes in PPAR signaling pathways were differentially expressed in BFMI mice.

Among the regulated genes, the expression of ubiquitin D (Ubd) was 15-fold higher in HFD in contrast to SMD. The expressions of *cathepsin S* (*Ctss*) *b* and *Col1a2* were also up-regulated (20-fold and 11-fold, respectively) in BFMI on HFD mice compared to SMD mice (6-fold and 2-fold, respectively).

DISCUSSION

This study aimed to identify genetic variances in the immune response to body fat content between BFMI and B6 mice, as well as to assess the influence of obesity on gene expression in inflammation-related pathways. We found that the expression of several genes related to the immune system was dysregulated in the adipocytokine signaling pathway and complement and coagulation cascades in the epididymal adipose tissue of BFMI mice on the HFD diet.

Adipocytokine Signaling Pathway

The results indicated that the response to dietary fat is influenced by genetic background (strain effect). The expressions of *Adipoq, Chuk, Ppara, Rxrg, Nfkbia, Socs3*, and *Slc2a1* were dysregulated in BFMI mice on HFD. Several studies in humans and rodents have provided evidence of the anti-diabetic, anti-atherogenic, and anti-inflammatory activities of Adipoq (10). Therefore, lower transcript levels of the Adipoq gene in BFMI mice may reduce adiponectin sensitivity, which could finally abet insulin resistance in BFMI vs. B6 mice. Low adiponectin transcript levels in the epididymal adipose tissue might be a key link the activation of the immune system in adipose tissue of BFMI males. The markedly decreased *Adipoq* expression in epididymal adipose tissue corresponds well with reduced serum adiponectin concentrations in BFMI mice on HFD. In healthy individuals, adiponectin suppresses the activation of TNF-α-mediated nuclear factor kappa B (NF-κB) (11). In addition, Nfkbia plays a role in the termination of NF-κB activity by binding to it (12). In our study, the down-regulation of *Adipoq* was in line with the up-regulation of *Nfkbia* and denoted the suppression of *NF-κB* activation.

Up-regulation of *Socs3*, a down-regulated target of *NF-κB*, supported this finding. As an important transcription factor, NF-κB regulates mediators of immune response, cell apoptosis, inflammation, embryonic development, and the cell cycle (11).

Socs3, which is also up-regulated in BFMI mice, suppresses cytokine signaling. Socs3 is a negative regulator of the insulin signaling pathway. It suppresses insulin-stimulated glucose uptake by inhibiting Irs1 in epididymal adipose tissue. This may lead to local insulin resistance (13). The higher leptin mRNA levels together with the up-regulated *Socs3* and reduced *Irs1* transcript levels in BFMI vs. B6 mice on HFD are in agreement with the study of Mori et al. (14), which showed that increased *Socs3* is a main regulator of diet-induced leptin and insulin resistance in Nestin-Cre and Synapsin1-Cre mice. Simultaneous up-regulation of *Socs3* and down-regulation of *Adipoq* transcript levels in BFMI indicate impaired insulin sensing and activation of local inflammation in adipose tissue in response to the HFD.

The up-regulation of fat-cell-specific glucose transporter 1 (*Slc2a1*) provides evidence for higher glucose uptake in epididymal adipose tissue of BFMI vs. B6 mice, particularly on HFD. Despite suppressed insulin-stimulated glucose uptake due to increased *Socs3* expression, this finding is consistent with studies on type 2 diabetic animals, which have also shown a significant increase in basal glucose uptake into adipose tissue as insulin-stimulated glucose uptake was inhibited (15).

The results suggest that the genes *Adipoq, Nfkbia*, and *Socs3* in the adipocytokine signaling pathway are most likely to link obesity to adipose-specific inflammation.

The diet effect was particularly notable in B6 mice on HFD. In this strain, glucose and fatty acid transport may be decreased, as indicated by the down-regulation of *Irs3* and *Cd36* expression, respectively. Adiponectin, an anti-inflammatory cytokine, inhibits the activation of NF-κB. Down-regulation of *Adipoq* together with *Irs3* and *CD36* expression may indicate the onset of inflammation in HFD-fed B6 mice. Gene expression was not influenced by diet in BFMI mice.

Complement and Coagulation Cascades

Our results showed that the genetic background (strain effect) significantly affected the alterations in gene expression caused by diet in the complement and coagulation cascades. The complement system bridges innate and acquired immunity and can be activated via three pathways: classical, lectin, and alternative (16). Genes of the classical and alternative pathways were activated in BFMI mice vs. B6. Among the factors of the complement cascade, the complement anaphylatoxins C3a and C5a play an important role in the metabolism of the entire body, energy balance, and the pathogenesis of diabetes and metabolic syndrome (17). They act as chemoattractants and trigger inflammation through their receptors C3ar1 and C5ar1 (16). These receptors have been identified as major positive regulators of insulin secretion, and their inhibitory effects on glucose-stimulated insulin secretion were demonstrated by Atanes et al. (17). Therefore, increased expression of *C3ar1* may imply that complement anaphylatoxins C3a and C5a activation could be in the early stage, and they may affect insulin secretion in BFMI vs. B6 mice on both SMD and HFD.

Complement Factor B (H2-Bf), which is also increased in BFMI mice, is an essential protein in the alternative pathway of complement activation. Moreno-Navarrete et al. (18) reported that increased expression of H2-Bf is associated with activation of the alternative complement pathway, which is linked to insulin resistance, obesity, and metabolic complications. The increased transcript levels of *H2-Bf* indicated that the alternative pathway, which promotes insulin resistance and atherosclerosis, was activated in the HFD group. Our results also

showed that the activity of the alternative pathway changed depending on the dietary effect.

The complement system interacts with the coagulation system (19). Tissue factor (TF, F3) is the main cellular initiator of the coagulation cascade and serves as a receptor for activated factor VII (FVIIa) and forms the TF-activated factor VII complex (20). The TF-activated factor VII complex mainly triggers coagulation as well as angiogenesis, inflammation, and atherosclerosis (20). Decreased *F3* expression level indicates that the coagulation cascade may not be activated in BFMI vs. B6 mice fed with SMD. The blood clotting factor F7 is important for the initiation of F3-induced extrinsic coagulation levels that are related to plasma triglyceride levels (21). The TF-activated factor VII complex activates protease-activated receptor 1 (F2r, PAR1), which contributes to adipogenesis and promotes inflammation and angiogenesis (22). Furthermore, a strong association was found between F2r and basal glucose levels in high-fat-fed mice (23). Plasminogen activator urokinase receptor (Plaur) contributes to macrophage infiltration in white adipose tissue (24). Therefore, increased transcript levels of Plaur and F2r may indicate macrophage migration and initiation of inflammation in the epididymal adipose tissue of BFMI mice on HFD.

The diet only significantly affected the expression of the *Adipsin* (*And*) gene in BFMI mice. Adn is an adipocyte serine protease that participates in triacylglycerol synthesis in human adipocytes, triggers insulin secretion, maintains β-cell function, and promotes adipocyte differentiation through C3a-C3aR signaling. Its expression is reduced in BFMI mice as well as in obese and diabetic animal models (25). In the current study, the up-regulated expression of *C3ar1* and down-regulated expression of *Adn* indicated that adipogenesis was underway in the animals and their energy balance was disturbed. Thbd is a cell surface-expressed glycoprotein that is involved in coagulation, fibrinolysis, complement activation, inflammation, and cell proliferation (26). Complement and coagulation cascades may not be activated as the expression of *F3, H2-Bf*, and *Thbd* is down-regulated in B6 mice. A recent study showed that dysregulated thrombin activity, including Thbd and fibrin, promotes obesity (27).

The differentially expressed genes *Nfatc4, Lat, Nfkb2* and *Nfkbia* in BFMI vs B6 on HFD vs. SMD indicate the effects of the interaction between strain and diet. Decreased *Irs3* and increased *Cd36, Cd3e*, and *Cd81* expressions were also detected in BFMI mice. In most cell types, NF-κB is inactive in the cytoplasm, likely because of the inhibitory effect of Nfkbia. The activation of NF-κB depends on the phosphorylation of Nfkbia by the inhibitor of κB (IκB) kinase (IKK). This modification leads to free NF-κB that translocates to the nucleus. It regulates the transcription of response genes encoding chemokines, cytokines, adhesion molecules, inflammation-associated enzymes and inhibitors of apoptosis (12). It can therefore be suggested that the up-regulation of *Nfkbia* in BFMI mice may enhance the inflammatory response in epididymal adipose tissue. The Nfat group of transcription factors is an essential component in cytokine gene expression upon T-cell activation. Kim et al. (28) reported that Nfatc4 and Atf3 negatively regulate adiponectin gene transcription. Thus, Nfatc4 may contribute to decreased adiponectin expression. Yang et al. (29) demonstrated that Nfatc4 contributes also to glucose and insulin metabolism. Increased *Nfatc4* expression in BFMI, together with reduced adiponectin concentration, may lead to increased insulin resistance. The linker for activation of T-cells (Lat) acts as a transmembrane scaffold protein critical for T-cell development and activation (30). Therefore, the up-regulation of *Lat* implies the activation of T-cells. Our results provide evidence that dysregulation of Nfkbia and Nfatc4 in particular may affect the activation of the signaling cascade of obesity-induced inflammation.

Irs3 is important in Glut4 translocation and glucose transport in primary adipocytes (31). Reduced *Irs3* transcript levels indicate decreased glucose transport, as indicated by up-regulated *Socs3* expression in the adipocytokine signaling pathway. Increased fatty acid transport in HFD was supported by the up-regulation of *CD36*, which facilitates fatty acid uptake into muscle and adipose tissue (32). Oguri et al. (33) found that loss of *CD81* causes diet-induced weight gain, glucose intolerance, insulin resistance, and adipose tissue inflammation. Thus, CD81 expression may be increased to prevent some metabolic disorders.

We also analyzed several pathways related to the immune response to provide a comprehensive assessment of inflammation. The genes exhibiting the most significant changes were identified in BFMI vs. B6 mice on HFD. Their gene expression profiles demonstrated that the number of significantly expressed genes associated with the immune system was higher in HFD than in SMD. In HFD, the regulated genes were detected only in the Toll-like receptor, B-cell receptor, and natural killer cell signaling pathways. These results indicate that these pathways may play a role in the differences between BFMI and B6 mice in terms of immune processes in adipose tissue.

The diet effect was significant only for the genes in the PPAR signaling pathways that showed differential expression in BFMI mice in HFD vs SMD.

The expression of *ubiquitin D* (*Ubd*) was up-regulated in BFMI on HFD compared with SMD. Ubd is involved in protein degradation and apoptosis (34) and mediates NF-kappaB activation. It is not currently annotated in a pathway. The expression of *cathepsin S* (*Ctss*) *b* and *Col1a2* was up-regulated in BFMI on SMD mice. The change in *Ctss* expression is noteworthy in terms of their pathological effects. Previous studies have reported that it provides a molecular link between obesity and the development of cardiovascular disease (35). Moreover, Hsing et al. (36) noted that Ctss deficiency led to decreased diabetes incidence. These results were expected given that BFMI has 4 times more fat stored in SMD compared to B6 at 10 weeks of age.

The results of the present study revealed clear differences between the two strains. In general, we observed more differentially expressed genes in BFMI vs. B6 fed an HFD. It is evident that the effect of HFD on the aforementioned systems (immune, endocrine signaling molecules and interaction and signal transduction system) in adipose tissue is more profound in BFMI than in B6 mice. In particular, genes associated with immune system pathways were significantly up-regulated in the epididymal adipose tissue of BFMI mice. The immune response signal could be attributed to adipose tissue macrophages, which are involved in the immune system. It has already been shown that obesity causes inflammatory changes in the body (16).

CONCLUSIONS

Our results show that increased fat deposition in adipose tissue activates signaling cascades leading to increased inflammatory gene expression in both strains. BFMI mice are more responsive to HFD with obesity than B6 mice. This may be due to the different genetic compositions of these strains, as BFMI is already obese on SMD in contrast to B6. It is expected that most processes involved in the inflammatory response are significantly up-regulated in the epididymal adipose tissue of BFMI mice as a result of obesity. It is noteworthy that genes involved in the adipocytokine signaling pathway and the complement and coagulation cascades were differentially expressed between BFMI and B6 mice fed the same diet. Genes in immune-related pathways were also differentially regulated. These results imply that genotype plays an important role in obesity-induced inflammation. Inflammatory adipokines or proteins appear to function significantly in the "low-grade inflammatory state" in BFMI on SMD and more so on HFD, but also in B6 mice on HFD. When they enter the circulation, a cluster of metabolic abnormalities, such as insulin resistance and activation of complement cascades, can be induced and may be linked to an increased risk of diabetes and atherosclerosis. In addition, the interaction between strain and diet was very likely to have an impact on the inflammatory signaling network. The implication for humans is that genetic predisposition is not the only contributor to disease risk factors, but that genetic predisposition together with diet may activate a critical pathway leading to increased disease risk and further manipulation of obesity.

Acknowledgements

We thank Helge Gössling for microarray experiments, Ralf Bortfeldt for support in bioinformatics, Asja Wagener, Reinhard Schiefler, Katrin Beck, and Ulf Kiesling for mouse husbandry, and Pamela Kepper for technical assistance. This research was supported by grants from the German National Genome Research Network (NGFN: 01GS0486, 01GS0829), grants from the German Research Foundation (GRK1209) and the German Network for Systems Genetics (GeNeSys).

Ethics Committee Approval: All experiments were performed in accordance with the approval of the German Animal Welfare Authorities (approval no. G0152/04 and V54-19c20/15c MR 17/1).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.B.; Data Acquisition: A.D., G.B.; Data Analysis/Interpretation: A.D., G.B.; Drafting Manuscript- A.D.; Critical Revision of Manuscript- G.B.; Final Approval and Accountability- A.D., G.B.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This research was supported by grants from the German National Genome Research Network (NGFN: 01GS0486, 01GS0829), grants from the German Research Foundation (GRK1209) and the German Network for Systems Genetics (GeNeSys).

REFERENCES

1. Phillips CL, Grayson BE. The immune remodel: Weight loss-mediated inflammatory changes to obesity. Exp Biol Med (Maywood) 2020; 245(2): 109-121.
2. Mikhailova SV, Ivanoshchuk DE. Innate-Immunity Genes in Obesity. J Pers Med 2021; 11(11): 1201.
3. Wagener A, Goessling HF, Schmitt AO, Mauel S, Gruber AD, Reinhardt R, et al. Genetic and diet effects on Ppar-α and Ppar-γ signaling pathways in the Berlin Fat Mouse Inbred line with genetic predisposition for obesity. Lipids Health Dis 2010; 9: 99.
4. Hantschel C, Wagener A, Neuschl C, Teupser D, Brockmann GA. Features of the metabolic syndrome in the Berlin Fat Mouse as a model for human obesity. Obes Facts 2011; 4(4): 270-7.
5. Reimers M, Carey VJ. Bioconductor: an open source framework for bioinformatics and computational biology. Methods Enzymol 2006; 411: 119-34.
6. Dunning MJ, Smith ML, Ritchie ME, Tavare S. beadarray: R classes and methods for Illumina bead-based data. Bioinformatics 2007; 23: 2183-4.
7. Ihaka R, Gentleman R. A language for data analysis and graphics. J Comp Graph Statistics 1996; 5: 299-314.
8. Cui X, Churchill GA. Statistical tests for differential expression in cDNA microarray experiments. Genome Biol 2003; 4: 210.
9. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 2002; 31**:** 19-20.
10. Von Frankenberg AD, Reis AF, Gerchman F. Relationships between adiponectin levels, the metabolic syndrome, and type 2 diabetes: A literature review. Arch Endocrinol Metab 2017; 61: 614–22.
11. Choi HM, Doss HM, Kim KS. Multifaceted physiological roles of adiponectin in inflammation and diseases. Int J Mol Sci 2020; 21(4): 1219.
12. Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2002; 109 Suppl**:** S81-96.
13. Wunderlich CM, Hövelmeyer N, Wunderlich FT. Mechanisms of chronic JAK-STAT3-SOCS3 signaling in obesity. JAKSTAT 2013; 2(2): e23878.
14. Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, et al. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med 2004; 10: 739-43.
15. Talior I, Yarkoni M, Bashan N, Eldar-Finkelman H. Increased glucose uptake promotes oxidative stress and PKC-delta activation in adipocytes of obese, insulin-resistant mice. Am J Physiol Endocrinol Metab 2003; 285: E295-302.

16. Al Haj Ahmad RM, Al-Domi HA. Complement 3 serum levels as a pro-inflammatory biomarker for insulin resistance in obesity. Diabetes Metab Syndr 2017; 11 Suppl 1: S229-32.
17. Atanes P, Ruz-Maldonado I, Pingitore A, Hawkes R, Liu B, Zhao M, et al. C3aR and C5aR1 act as key regulators of human and mouse β-cell function. Cell Mol Life Sci 2018; 75(4): 715-26.
18. Moreno-Navarrete JM, Martínez-Barricarte R, Catalán V, Sabater M, Gómez-Ambrosi J, Ortega FJ, et al. Complement factor H is expressed in adipose tissue in association with insulin resistance. Diabetes 2010; 59(1): 200-9.
19. Shim K, Begum R, Yang C, Wang H. Complement activation in obesity, insulin resistance, and type 2 diabetes mellitus. World J Diabetes 2020;11(1):1-12.
20. Bernardi F, Mariani G. Biochemical, molecular and clinical aspects of coagulation factor VII and its role in hemostasis and thrombosis. Haematologica 2021; 106(2): 351-62.
21. Morrissey JH. Tissue factor interactions with factor VII: measurement and clinical significance of factor VIIa in plasma. Blood Coagul Fibrinolysis 1995; 6 Suppl 1: S14-19.
22. Yi X, Wu P, Liu J, Gong Y, Xu X, Li W. Identification of the potential key genes for adipogenesis from human mesenchymal stem cells by RNA-Seq. J Cell Physiol 2019; 234(11): 20217-27.
23. Macias-Velasco JF, St Pierre CL, Wayhart JP, Yin L, Spears L, Miranda MA, et al. Parent-of-origin effects propagate through networks to shape metabolic traits. Elife 2022; 11: e72989.
24. Cancello R, Rouault C, Guilhem G, et al. Urokinase plasminogen activator receptor in adipose tissue macrophages of morbidly obese subjects. Obes Facts 2011; 4(1): 17-25.
25. Song NJ, Kim S, Jang BH, Chang SH, Yun UJ, Park KM, et al. Small molecule-induced complement factor D (Adipsin) promotes lipid accumulation and adipocyte differentiation. PLoS One 2016; 11(9): e0162228.
26. Van de Wouwer M, Plaisance S, De Vriese A, Waelkens E, Collen D, Persson J, et al. The lectin-like domain of thrombomodulin interferes with complement activation and protects against arthritis. J Thromb Haemost 2006; 4: 1813-24.
27. Kopec, AK, Abrahams SR, Thornton S, Palumbo JS, Mullins ES, Divanovic S, et al. Thrombin promotes diet-induced obesity through fibrin-driven inflammation. J Clin Invest 2017; 127(8): 3152-66.
28. Kim HB, Kong M, Kim TM, Suh YH, Kim WH, Lim JH, et al. NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes. Diabetes 2006; 55: 1342-52.
29. Yang TTC, Suk HY, Yang XY, Olabisi O, Yu RYL, Durand J, et al. Role of transcription factor NFAT in glucose and insulin homeostasis. Mol Cell Biol 2006; 26: 7372-87.
30. Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 1998; 92: 83-92.
31. Zhou L, Chen H, Xu P, Cong LN, Sciacchitano S, Li Y, et al. Action of insulin receptor substrate-3 (IRS-3) and IRS-4 to stimulate translocation of GLUT4 in rat adipose cells. Mol Endocrinol 1999; 13: 505-14.
32. Son NH, Basu D, Samovski D, Pietka TA, Peche VS, Willecke F, et al. Endothelial cell CD36 optimizes tissue fatty acid uptake. J Clin Invest 2018; 128(10): 4329-42.
33. Oguri Y, Shinoda K, Kim H, Alba DL, Bolus WR, Wang Q, et al. CD81 controls beige fat progenitor cell growth and energy balance via FAK signaling. Cell 2020; 182(3): 563-77.e20.
34. Canaan A, Yu X, Booth CJ, Lian J, Lazar I, Gamfi SL, et al. FAT10/diubiquitin-like protein-deficient mice exhibit minimal phenotypic differences. Mol Cell Biol 2006; 26: 5180-9.
35. Rodgers KJ, Watkins DJ, Miller AL, Chan PY, Karanam S, Brissette WH, et al. Destabilizing role of cathepsin S in murine atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2006; 26: 851-6.
36. Hsing LC, Kirk EA, McMillen TS, Hsiao SH, Caldwell M, Houston B, et al. Roles for cathepsins S, L, and B in insulitis and diabetes in the NOD mouse. J Autoimmun 2010; 34: 96-104.

Experimed 2024; 14(2): 85-91

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1461284

Altered Tissue Factor Activity and Disrupted Oxidant-Antioxidant Status in Saliva of Patients with Oral Lichen Planus

Derya Cansiz[1], Merih Beler[2], Ismail Unal[1,2], Gizem Egilmezer[2], Zulal Mizrak[2], Semanur Isikoglu[2], Efruz Irem Akkus[3], Birsay Gumru[4], Sebnem Ercalik Yalcinkaya[4], Ebru Emekli-Alturfan[5]

[1]Department of Biochemistry, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkiye
[2]Institute of Health Sciences, Marmara University, Istanbul, Turkiye
[3]Department of Medical Biology, Faculty of Medicine, Istanbul University-Cerrahpasa, Istanbul, Turkiye
[4]Department of Oral and Maxillofacial Radiology, Faculty of Dentistry, Marmara University, Istanbul, Turkiye
[5]Department of Biochemistry, Faculty of Dentistry, Marmara University, Istanbul, Turkiye

ORCID ID: D.C.: 0000-0002-6274-801X; M.B.: 0000-0002-3828-4630; I.U.: 0000-0002-8664-3298; G.E.: 0000-0002-1231-5232; Z.M.: 0009-0004-7647-7267; S.I.: 0009-0003-9779-2867; E.I.A.: 0000-0001-8504-2602; B.G.: 0000-0002-7734-4755; S.E.Y.: 0000-0003-2924-1935; E.E.A.: 0000-0003-2419-8587

Cite this article as: Cansiz D, Beler M, Unal I, Egilmezler G, Mizrak Z, Isikoglu S, Akkus EI, Gumru B, Ercalik Yalcinkaya S, Emekli Alturfan E. Altered tissue factor activity and disrupted oxidant-antioxidant status in saliva of patients with oral lichen planus. Experimed 2024; 14(2): 85-91.

ABSTRACT

Objective: The aim of our study was to investigate salivary tissue factor (TF) activity and oxidant-antioxidant status, which is an important defense system, in saliva samples collected from patients with oral lichen planus (OLP), and to determine the relationship between the antioxidant system and TF activity in OLP.

Materials and Methods: Saliva samples were collected from patients with OLP (n=20) and healthy subjects (n=13). TF activity, lipid peroxidation (LPO), nitric oxide (NO), glutathione (GSH) levels, glutathione S-transferase (GST), and superoxide dismutase (SOD) activities were measured.

Results: NO levels and GST activities were increased, whereas GSH levels and SOD activities were decreased, when compared with healthy subjects in the saliva samples collected from patients with OLP. In addition, TF activity was increased in the OLP group compared with the control group

Conclusion: The results revealed that the salivary oxidant-antioxidant balance was disrupted in parallel with the increase in TF activity in patients with OLP.

Keywords: Oral lichen planus, tissue factor, oxidative stress, saliva

Corresponding Author: Derya Cansiz **E-mail:** cansizderya@yahoo.com
Submitted: 01.04.2024 **Revision Requested:** 24.04.2024 **Last Revision Received:** 17.05.2024 **Accepted:** 24.05.2024

INTRODUCTION

Lichen planus (LP) is a chronic inflammatory disease commonly observed in the skin and mucosa, the cause of which is unknown. Oral lichen planus (OLP), which is most commonly seen in the buccal mucosa, dorsum of the tongue, and less commonly on the gingivae, can occur either alone or with skin lesions, and is usually bilateral and symmetrical (1, 2). The mouth and saliva reflect physiological or pathological changes in the organism (3). Saliva, with its rich content, can be used to diagnose various diseases or monitor the body's response to treatment. Saliva biomarkers are important for determination of these changes in the body. These biomarkers play a potential role in the diagnosis of various oral and systemic diseases. In addition, analysis with saliva fluid provides an advantage because it is an non-invasive method (3, 4).

Tissue factor (TF, Factor III, Thromboplastin) is an important activator of the coagulation cascade, and is the main regulator of haemostasis and thrombosis (5). Body fluids such as saliva, bile, semen, amniotic fluid, and various tissues have TF activity (6-9). The clotting function of saliva is due to TF present in saliva, and TF activity may be triggered by many different stimuli, including surface expression, inflammatory cytokines, endotoxins, and hypoxia (6, 7, 10).

In the presence of oxygen under normal cell conditions, reactive oxygen species (ROS) can be produced. The increase in ROS levels compared with antioxidant agents causes the oxidant-antioxidant balance to deteriorate. This imbalance causes various diseases because of lipid peroxidation (LPO) and cell damage (5, 6).

Oxidant-antioxidant imbalance has a potential effect on the development of oral pathologies. Many diseases are based on oxidative stress, which can also explain why the systemic and oral disease consequences can be so severe. Antioxidants, which are effective in the defense mechanism of the organism, are also included in the saliva content (4-6). LPO and salivary antioxidants can be utilized to measure the oral oxidant-antioxidant condition (5, 6, 11-13).

Free radicals produced by exogenous or endogenous sources in the organism react with lipids and cause LPO, which can have a very harmful effect on the organism. LPO causes the formation of toxic products that show harmful effects in areas far from where they are produced (12). Malondialdehyde (MDA) is the major end product of LPO and is recognized as an important marker of oxidative stress and damage mediated by free radicals (13). The antioxidant system, which is effective as a defense mechanism in the organism, consists of enzymatic and non-enzymatic agents. Glutathione (GSH), which has a tripeptide structure, is a non-enzymatic antioxidant. GSH, which has an antioxidant effect, is a reducing agent. GSH serves as a cofactor for enzymes such as glutathione peroxidase and glutathione S-transferase (GST). In addition, GSH plays a role in the regeneration of vitamin E for the detoxification of lipid peroxyl radicals. GST are enzymes involved in the catalysis of the reaction between the reducing agent GSH and xenobiotics. The key role of the GST enzyme in xenobiotic metabolism is to prevent cell damage by combining the reactive species produced by the enzymes in Phase I with glutathione (14).

Superoxide dismutase (SOD) catalyzes the conversion of superoxide radicals, a by-product of oxygen metabolism, into molecular oxygen and hydrogen peroxide. The primary antioxidant enzyme systems SOD and glutathione peroxidase can destroy free radicals. OLP develops as a result of increased oxidative stress (13). Because salivary fluid naturally contains various antioxidants, many studies have been conducted using salivary fluid, and these studies have revealed that the total antioxidant status in salivary fluid collected from erosive OLP patients is lower than that in healthy controls (14, 15). Nitric oxide (NO), which is synthesized from arginine, play roles as a neurotransmitter, immunomodulator, vasodilator, and antiplatelet. The neuronal nitric oxide synthase (nNOS) enzyme, which catalyzes NO synthesis, is located in the salivary gland. Therefore, it is thought that NO may play a potential role in salivary gland secretion and blood flow. In addition, because NO has antibacterial properties, it can protect the oral mucosa at low concentrations in saliva (16).

ROS may interfere with platelet activation and increase the expression levels of TF in endothelial cells, monocytes, and vascular smooth muscle cells, all of which can promote coagulation (17). In view of the ability of ROS to induce oxidative structural as well as functional alterations in key proteins involved in the coagulation cascade, the aim of our study was to investigate salivary TF activity and oxidant-antioxidant parameters to evaluate the relationship between TF activity and oxidant-antioxidant status in patients with OLP.

MATERIALS AND METHODS

Saliva Collection and Storage

The Istanbul Medipol University Non-invasive Clinical Research Ethics Committee approved the study protocol (ethic no: E-10840098-202.3.02-986). All oral clinical examinations were performed at the Outpatient Clinic of the Department of Oral and Maxillofacial Radiology, Faculty of Dentistry, Marmara University.

Patients with OLP whose disease was confirmed by clinical examination by two specialists with more than 20 years of experience based on clinical manifestations (reticular lesions alone or in association with erosive/ulcerative lesions) or combined with histopathological examination were included in the current study.

After the selection of participants, unstimulated saliva samples were collected from 20 OLP patients (aged 30-55 years), and 13 healthy controls using the spitting method. Participants were instructed to stop eating, drinking, and brushing their teeth 90 min before saliva samples were collected. The patient

must sit in a comfortable position while the saliva sample was being collected. Leaning slightly forward, the patient drained the saliva from the tube by spitting 1-2 times every 1 min for 5 min. After saliva collection, the test tube was sealed with a plastic cap and transported to the biochemistry laboratory. Whole saliva samples were used to assess tissue factor activity. For biochemical parameters, saliva samples were centrifuged at 825xg for 4-5 minutes and the supernatant was separated and used in the analysis. The analyses were performed in a blinded manner.

Saliva Analysis

Assay of TF Activity

Quick's one-step method was used to measure TF activity in saliva samples (18). This was accomplished by addition of 100 µL of 0.02 M $CaCl_2$ to 100 µL of saliva and 100 µL of plasma to initiate the coagulation reaction. Activity was measured after the temperatures of all solutions used in the experiment were brought to 37°C. Because clotting time is inversely proportional to TF activity, prolonged clotting time is an indicator of decreased TF activity.

Assay of the Total Protein

Protein levels were measured to present biochemical data as values per mg of protein. Proteins react with Cu^{2+} ions in an alkaline environment. It is then reduced with the foline reagent. The intensity of the resulting color was evaluated spectrophotometrically. The density of the colored (blue-violet) solution formed is proportional to the protein concentration (19).

Assay of Lipid Peroxidation

LPO determination was made by spectrophotometric evaluation at 532 nm of the density of the coloured solution resulting from the reaction between the LPO product MDA and thiobarbituric acid (20).

Assay of NO

The determination of NO was performed by spectrophotometric measurement at 540 nm of the colored product resulting from the formation of a complex diazonium compound by the reaction of nitritesulfanilamide with N-(1-Naphthyl) ethylenediaminedihydrochloride in an acidic environment (21).

Assay of the GST Activity

The determination of GST activity was evaluated spectrophotometrically by measuring the absorbance of the product formed after the conjugation of GSH with 1-chloro-2,4-dinitro-benzene at 340 nm (22).

Assay of the SOD Activity

SOD activity is measured as the ability to increase the photooxidation rate of riboflavin- sensitized o-dianisidine. The superoxide radical formed by riboflavin under the influence of fluorescence light turns into hydrogen peroxide under the influence of SOD in the environment. Hydrogen peroxide reacts with o-dianisidine to form a colored product. The absorbance of the resulting colored product was evaluated spectrophotometrically at 460 nm (23).

Assay of GSH

GSH determination was made spectrophotometrically by the colored product resulting from the reaction of Elmann's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and sulfhydryl groups (24).

Statistical Analyses

GraphPad Prism 9.0 was used for statistical analysis, and the mean and standard deviation (SD) was presented as the result. An unpaired samples t-test was performed to compare the control and OLP groups.

Results

When we examined TF activity to measure the coagulative function of saliva and compared it with the control group, increased salivary TF activity was determined in patients with OLP (Figure 1).

In the LPO analysis, where we measured MDA levels, there was no statistically significant difference in MDA levels (Figure 2A), and NO levels were raised in the patients with OLP compared with the control group (Figure 2B).

While GST activity was increased statistically in patients with OLP compared with the control (Figure 3A), SOD activity (Figure 3B), and GSH levels (Figure 3C) were decreased.

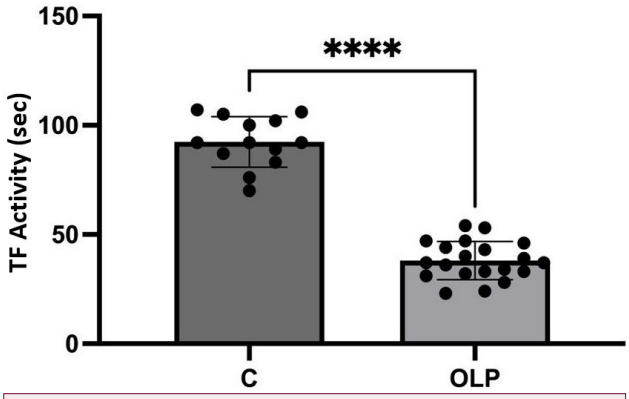

Figure 1. Tissue Factor (TF) activities of the groups. Clotting time is inversely proportional to TF activity. The longer clotting time in seconds is a manifestation of lower TF activity. Data are given as mean ± SD, **** p<0.0001, SD: Standart deviation, C: Control group; OLP: Oral Lichen Planus

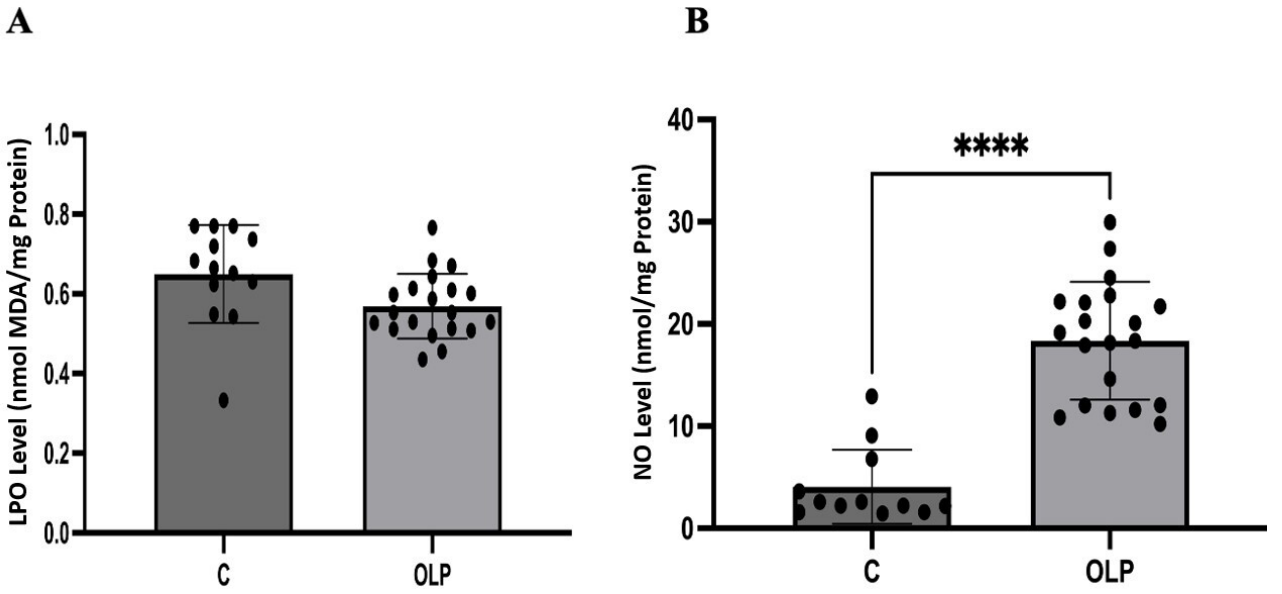

Figure 2 A. MDA levels serve as an index of lipid peroxidation (LPO). **B**. Nitric Oxide (NO) levels. Data are given as mean ± SD, **** p<0.0001, SD: Standart deviation, C: Control group; OLP: Oral Lichen Planus

DISCUSSION

The body has specific mechanisms called antioxidant defense systems to deal with free radical damage. Increased cellular damage is caused by oxidative stress or a failure of the antioxidant defense system (25-29). Various antioxidant parameters (such as GST and SOD activity) are analyzed to determine the level of oxidative stress.

Inflammatory cellular infiltrates caused by $CD4^+$ lymphocytes are a potential source of reactive oxygen species. Increased oxidative stress has previously been reported in patients with OLP, a chronic inflammatory disease (25-29). Consistent with these studies, in our study the oxidant-antioxidant balance was disturbed, characterized by decreased antioxidant enzyme activities; however, we did not find any statistical difference between the control group and the OLP patient group.

In our study, changes in oxidant-antioxidant parameters and TF activity were analyzed in saliva samples collected from patients with OLP. Consistent with our results, Tunali-Akbay et al. showed that TF activity was increased in saliva samples of patients with OLP (29). Our results showed that the oxidant-antioxidant balance was disrupted and salivary TF activity was increased in patients with OLP. Over the past few years, oxidative stress has become increasingly important in controlling endothelial dysfunction and thrombus development. ROS may hinder the coagulation process through various intricately linked pathways. ROS, mostly produced by NADPH oxidase (NOX) enzymes, can directly induce the coagulation cascade by upregulating TF expression in various cells such as monocytes and endothelial cells. By oxidatively altering proteins implicated in the coagulation process, ROS can also induce a procoagulant situation (17).

TF is the activator of the extrinsic pathway of the coagulation cascade, and its activity is sensitive to changes in membrane content, temperature, and pH (29). The increase in TF activity is related to the activation of the coagulative functions of tissues. In our study, we suggest that this situation is triggered by increased oxidative stress in the OLP. The increase in TF activity appears to be significant in OLP, where inflammation is important in pathogenesis. An autoimmune reaction is often suggested to be the cause of OLP, but some experts argue that microbial infection initiates the autoimmune response (30). Salivary cells are responsible for approximately 78% of the TF activity in saliva (9). Emekli-Alturfan et al. (6) demonstrated an inverse relationship between salivary TF activity and bacterial cell count, and suggested that the storage time of saliva is the reason for this negative association.

In our study, although no difference in LPO levels was found in the saliva samples of OLP patients, the increase in NO as a level of oxidative stress was notable. NO is an important free radical, and has proinflammatory and cytotoxic effects on human skin (25). Our result is compatible with the findings of Mehdipour et al. (31) who revealed increased NO levels in serum samples collected from patients with OLP, and suggested the activation of lymphocytes and the cellular immune system as a consequence. By increasing oxidant production and lowering antioxidant protection, the elevated NO in saliva may cause oxidative stress on reactive nitrogen species, which would tangentially relate to OLP. In addition, elevated NO causes tissue and cell damage, and studies have shown that elevated NO can damage oral epithelial cells, keratinocyte, and fibroblasts (32).

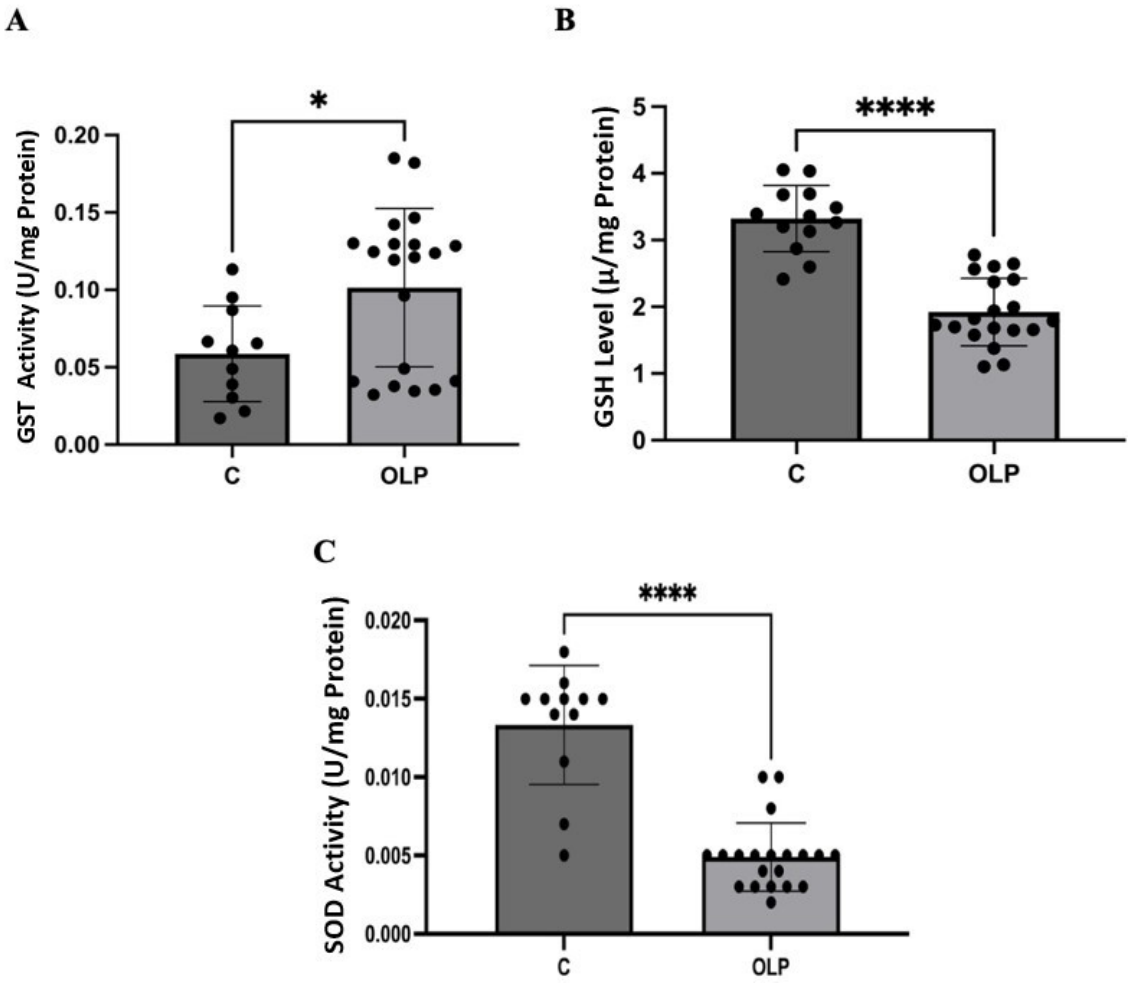

Figure 3 A. Glutathione S-transferase activities of the groups **B**. Superoxide dismutase (SOD) activities of the groups **C**. Glutathione (GSH) levels in the groups. Data are given as mean ± SD, * p<0.05; **** p<0.0001, SD: Standart deviation, C: Control group; OLP: Oral Lichen Planus

Increased oxidative stress in patients with OLP seriously compromises the integrity of cells by interfering with membrane fluidity, organization, crosslinking, and functioning and may influence the interactions between Bcl-2 and Bax, which in turn affects signal transmission and apoptosis (1, 33). The apoptotic cascade triggered by mitochondrial apoptotic signaling and oxidative stress begins with a decrease in cellular GSH content (34). In our study, the decreased GSH levels in the saliva samples of patients with OLP may be related to the triggering of apoptotic processes in these patients. Similar to our results, Hassan et al. revealed a decline in GSH levels in patients with OLP (25). Decreased GSH levels may cause a boost in hydrogen peroxide levels, which may damage the basal cell layer (25).

An array of electrophilic compounds can be conjugated with GSH through the action of GST enzymes, whose most well-known function is that of cell housekeepers involved in xenobiotic detoxification. It has recently been shown that GSTs also function as regulators of pathways of signaling that regulate the growth and death of cells. GST provides a protective effect against various toxic chemicals in cells (14). In contrast to the increased GST activity in our study, Jana et al. (28) reported decreased GST activity in OLP patients and suggested that the erosive LP is more under stress than the reticular form. The reason for the difference in the results of these two studies may be the low number of severe (erosive/ulcerative and atrophic) cases in our study. This may also be due to the triggering of the GST defense mechanism against increased oxidative damage.

ROS triggers pro-inflammatory and oxidative stress-associated conditions, as well as DNA damage, which further activates signaling pathways leading to cell death and apoptosis, creating a vicious cycle of damage (33). The SOD enzyme catalyzes the formation of hydrogen peroxide and molecular oxygen from superoxide radicals, making them less harmful. We found a decrease in SOD activity, which is the first defense

enzyme in the antioxidant system against oxidative stress, in patients with OLP. Similar to our findings, Shirzaiy et al. (35) reported decreased SOD activity in patients with OLP. However, Hassan et al. showed an increase in SOD activity in patients with OLP (25). It is useful to note that the decrease in LPO levels in our study may have been prevented by the SOD enzyme, leading to its consumption and decrease.

We propose that the alteration in TF activity may be related to the disturbed oxidant-antioxidant balance based on the data obtained in this study. Although our study supports the diagnostic importance of saliva in OLP disease, we believe that further studies involving the investigation of the hemostatic system and coagulation factors in the immune, ROS, inflammatory, and apoptosis-related pathways of OLP will clarify this issue.

The limited sample size and the absence of analysis of inflammatory cytokines in saliva are among the limitations of our study. In future studies, determination of the inflammatory cytokines and oxidative stress markers in saliva at both protein and gene levels and comparing them with the clinical findings of patients will contribute to elucidating the molecular mechanism of OLP.

Acknowledgment: We would like to express our gratitude to Great Leader Mustafa Kemal Atatürk and his comrades in arms, who gave us the Republic. Happy 100th anniversary of the Republic of Turkiye.

Ethics Committee Approval: The Istanbul Medipol University Non-invasive Clinical Research Ethics Committee approved the study protocol (ethic no: E-10840098-202.3.02-986).

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- D.C., S.E.Y., B.G., E.E.A.; Data Acquisition: M.B., I.U., G.E., Z.M., S.I., E.I.A.; Data Analysis/Interpretation: D.C., S.E.Y., B.G., E.E.A.; Drafting Manuscript- M.B., I.U., G.E., Z.M., S.I., E.I.A.; Critical Revision of Manuscript- D.C., S.E.Y., B.G., E.E.A.; Final Approval and Accountability- D.C., S.E.Y., B.G., E.E.A., M.B., I.U., G.E., Z.M., S.I., E.I.A.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: The authors declare that this study has received no financial support.

REFERENCES

1. Erçalık Ş, Gümrü-Tarçın B. Oral liken planus ve güncel tedavi yaklaşımları: Bölüm 8. Sağlık Bilimlerinde Güncel Araştırmalar Cilt 2. Eds. Cem Evereklioğlu C, Erten M. Gece Kitaplığı. 2022.p. 97-112.
2. Gumru B. A retrospective study of 370 patients with oral lichen planus in Turkey. Med Oral Patol Oral Cir Bucal 2013; 18(3): e427-32.
3. Streckfus CF, Bigler LR. Saliva as a diagnostic fluid. Oral Dis 2002; 8(2): 69-76.
4. Lee JM, Garon E, Wong DT. Salivary diagnostics. Orthod Craniofac Res 2009; 12(3): 206-11.
5. Alturfan AA, Alturfan EE, Dariyerli N, Zengin E, Aytac E, Yigit G, et al. Investigation of tissue factor and other hemostatic profiles in experimental hypothyroidism. Endocrine 2006; 30: 63–7.
6. Emekli-Alturfan E, Kasikci E, Alturfan AA, Pisiriciler R, Yarat A. Effect of sample storage on stability of salivary glutathione, lipid peroxidation levels, and tissue factor activity. J Clin Lab Anal 2009; 23: 93-8.
7. Emekli-Alturfan E, Basar I, Malali E, Elemek E, Oktay S, Ayan F, et al. Plasma tissue factor levels and salivary tissue factor activities of periodontitis patients with and without cardiovascular disease. Pathophysiol Haemost Thromb 2010; 37(2-4): 77-81.
8. Yarat A, Tunali T, Pisiriciler R, Akyuz S, Ipbuker A, Emekli N. Salivary thromboplastic activity in diabetics and healthy controls. Clin Oral Invest 2004; 8: 36–9.
9. Zacharski LR, Rosenstein R. Reduction of salivary tissue factor (Thromboplastin) activity by warfarin therapy. Blood 1979; 53: 366–74.
10. Emekli-Alturfan E, Demir G, Kasikci E, Tunali-Akbay T, Pisiriciler R, Caliskan E, et al. Altered biochemical parameters in the saliva of patients with breast cancer. Tohoku J Exp Med 2008; 214: 89-96.
11. Rekha VR, Sunil S, Rathy R. Evaluation of oxidative stress markers in oral lichen planus. J Oral Maxillofac Pathol 2017; 21: 387–93.
12. Darczuk D, Krzysciak W, Vyhouskaya P, Kesek B, Galecka-Wanatowicz D, Lipska W, et al. Salivary oxidative status in patients with oral lichen planus. J Physiol Pharmacol 2016; 67: 885-94.
13. Bonola-Gallardo I, Irigoyen-Camacho ME, Vera-Robles L, Campero A, Gómez-Quiroz L. Enzymatic activity of glutathione S-transferase and dental fluorosis among children receiving two different levels of naturally fluoridated water. Biol Trace Elem Res 2017; 176(1): 40-7.
14. Agha-Hosseini F, Mirzaii-Dizgah I, Farmanbar N, Abdollahi M. Oxidative stress status and DNA damage in saliva of human subjects with oral lichen planus and oral squamous cell carcinoma. J Oral Pathol Med 2012; 41: 736-40.
15. Mansourian A, Agha-Hosseini F, Kazemi HH, Mortazavi N, Moosavi MS, Beytollahi J et al. Salivary oxidative stress in oral lichen planus treated with triamcinolone mouthrinse. Dent Res J 2017; 14: 104-10.
16. Humberto JSM, Pavanin JV, Rocha MJAD, Motta ACF. Cytokines, cortisol, and nitric oxide as salivary biomarkers in oral lichen planus: a systematic review. Braz Oral Res 2018; 32: e82.
17. Bettiol A, Galora S, Argento FR, Fini E, Emmi G, Mattioli I, et al. Erythrocyte oxidative stress and thrombosis. Expert Rev Mol Med 2022; 24: e31
18. Ingram GI, Hills M. Reference method for the one-stage prothrombin time test on human blood. Thromb Haemostas 1976; 36: 237–8
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.
20. Yagi K. Assay for blood plasma or serum. Methods Enzymol 1981; 105: 328-37.
21. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001; 5(1): 62-71.
22. Habig WH, Pabst MJ, & Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974; 249(22): 7130–9.
23. Mylorie AA, Collins H, Umbles C, Kyle J. Erythrocyte SOD activity and other parameters of copper status in rats ingesting lead acetate. Toxicol Appl Pharmacol 1986; 82: 512-20.

24. Beutler E. Gluthatione: Red cell metabolism a manual biochemical methods, 2nd ed. New York: Grune and Stratton, 1975. p.112–4.
25. Hassan I, Keen A, Majid S, Hassan T. Evaluation of the antioxidant status in patients of lichen planus in Kashmir valley–A hospital based study. J Saudi Soc Dermatol Dermatol Surg 2013; 17: 13–6.
26. Vlková B, Stanko P, Minárik G, Tóthová L, Szemes T, Baňasová L, et al. Salivary markers of oxidative stress in patients with oral premalignant lesions. Arch Oral Biol 2012; 57: 1651–6.
27. Ergun S, Troşala SC, Warnakulasuriya S, Ozel S, Onal AE, Ofluoglu D, et al. Evaluation of oxidative stress and antioxidant profile in patients with oral lichen planus. J Oral Pathol Med 2011; 40: 286–93.
28. Jana A, Thomas J, Ghosh P. Erosive oral lichen planus inflicts higher cellular stress than reticular type. J Oral Maxillofac Pathol 2021; 25(2): 279-85.
29. Tunali-Akbay T, Solmaz Z, Namdar Pekiner F, İpekci H. Salivary tissue factor concentration and activity in patients with oral lichen planus. Oral Sci Int 2017; 14(1): 13-7.
30. Villa TG, Sánchez-Pérez Á, Sieiro C. Oral lichen planus: a microbiologist point of view. Int Microbiol 2021; 24(3): 275-89.
31. Mehdipour M, Taghavi Zenouz A, Bahramian A, Gholizadeh N, Boorghani M. Evaluation of serum nitric oxide level in patients with oral lichen planus. J Dent (Shiraz) 2014; 15(2): 48-51.
32. Alamir AWH, Arakeri G, Patil S, Awan KH, Kujan O, Aljabab A, et al. Association of nitric oxide with oral lichen planus. J Oral Pathol Med 2019; 48(5): 345-50
33. Li X, Wang Z. Mechanisms of reactive oxygen species in oral lichen planus: A literature review. Europ J Inflam 2022; 20:1-9
34. Circu ML, Aw TY. Glutathione and apoptosis. Free Radic Res 2008; 42(8): 689-706.
35. Shirzaiy M, Salehian MA, Dalirsani Z. Salivary antioxidants levels in patients with oral lichen planus. Indian J Dermatol 2022; 67(6): 651-6.

Experimed 2024; 14(2): 92-101

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1472096

Relationship Between BDNF and LPS Levels in the Blood of Patients with Different Neurological Diseases: A Small Cohort Study

Nur Damla Korkmaz[1,2,3], Birsen Elibol[4], Seda Susgun[3], Ceyhun Toruntay[5], Alisan Bayrakoglu[6], Mazlum Yuzgulec[7], Zihni Elagoz[7], Aysegul Yabaci Tak[8], Emrah Yucesan[9], Ferda Ilgen Uslu[6], Gulsen Babacan Yildiz[6], Azize Esra Basar Gursoy[10], Fahri Akbas[3], Bilge Sumbul[11]

[1]Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkiye
[2]Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Turkiye
[3]Department of Medical Biology, Bezmialem Vakif University, Faculty of Medicine, Istanbul, Turkiye
[4]Department of Medical Biology, Istanbul Medeniyet University, Istanbul, Turkiye
[5]Department of Molecular Biology and Genetics, Faculty of Science and Letters, Istanbul Technical University, Istanbul, Turkiye
[6]Department of Neurology, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkiye
[7]Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkiye
[8]Department of Biostatistics and Medical Informatics, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkiye
[9]Department of Neurogenetics, Institute of Neurological Sciences, University-Cerrahpasa, Istanbul, Turkiye
[10]Department of Neurology, Basaksehir Cam and Sakura City Hospital, Istanbul, Turkiye
[11]Department of Medical Microbiology, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkiye

ORCID ID: N.D.K. 0000-0002-1173-1701; B.E. 0000-0002-9462-0862; S.S. 0000-0001-9689-3111; C.T. 0000-0002-4743-0257; A.B. 0000-0001-9620-2237; M.Y. 0009-0006-8949-0529; Z.E. 0009-0006-0517-8133; A.Y.T. 0000-0002-5813-3397; E.Y. 0000-0003-4512-8764; F.I.U. 0000-0002-2124-5037; G.B.Y. 0000-0003-0922-0969; A.E.B.G. 0000-0002-8103-0927; F.A. 0000-0002-3837-250X; B.S. 0000-0002-8768-3777

Cite this article as: Korkmaz ND, Elibol B, Susgun S, Toruntay C, Bayrakoglu A, Yuzgulec M, Elagoz Z, Yabaci Tak A, Yucesan E, Ilgen Uslu F, Babacan Yildiz G, Basar Gursoy AE, Akbas F, Sumbul B. Relationship between BDNF and LPS levels in the blood of patients with different neurological diseases: a small cohort study. Experimed 2024; 14(2): 92-101.

ABSTRACT

Objective: Neuroinflammation and blood-brain barrier (BBB) dysfunction are key factors in various neurological disorders, disrupting brain tissue balance and leading to neuronal death. BBB integrity decline is evident in Alzheimer's Disease (AD), Parkinson's Disease (PD), Multiple Sclerosis (MS), and epilepsy.

Materials and Methods: We measured levels of lipopolysaccharide (LPS), the largest endotoxin, and brain-derived neurotrophic factor (BDNF) in patients' blood plasma and correlated them with biochemical parameters to identify biomarkers for these diseases.

Results: Significant associations were observed between LPS, C-reactive protein (CRP), BDNF, and lactate dehydrogenase (LDH) levels across conditions. LPS was positively correlated with CRP levels in epilepsy ($r=0.753$, $p=0.002$). Additionally, BDNF was negatively correlated with CRP in PD patients ($r=-0.53$, $p=0.042$). Moreover, a negative correlation was found between LPS and LDH in AD patients ($r=-0.521$, $p=0.047$).

Conclusion: Our findings correspond to the etiology of neuroinflammation involved in the pathophysiology of relevant diseases and suggest the potential use of these biomarkers in the early diagnosis and monitoring of neurological diseases, guiding future research towards better patient outcomes and therapies.

Keywords: Brain-derived neurotrophic factor, lipopolysaccharide, neuroinflammation, neurological diseases, biomarker

Corresponding Author: Birsen Elibol **E-mail:** elibolbirsen@gmail.com
Submitted: 25.04.2024 **Revision Requested:** 11.06.2024 **Last Revision Received:** 17.06.2024 **Accepted:** 16.07.2024

INTRODUCTION

The molecular origins of most neurological diseases and disorders are still largely obscure. However, most previous studies have shown that neuroinflammation and blood-brain barrier (BBB) dysfunction are at the root of a wide variety of neurological disorders and are associated with disease progression (1, 2). Although the inflammatory response process in the central nervous system (CNS) might play an active role in the healing of damaged tissue, irregular inflammatory processes have been shown to disrupt homeostasis of brain tissue and cause neuronal death (3).

The most important component of the clinical picture of neuroinflammation-mediated neurodegeneration is the deterioration of BBB structure and function (4). According to the findings obtained from the studies, BBB integrity was impaired and the neurovascular unit structure was destroyed in neurodegenerative diseases such as Alzheimer's Disease (AD) (5), Parkinson's disease (PD) (6), Multiple Sclerosis (MS) (7), and epilepsy (8). In this context, lipopolysaccharide (LPS), the largest endotoxin, may be an important tool for evaluating the neuroinflammation and BBB integrity axis. Briefly, when bacteria die, endotoxins are generally released, creating an antigenic effect that stimulates myeloid cells and initiates the neuroinflammation process, respectively (9, 10). For instance, studies on the endotoxin hypothesis in neurodegeneration have shown that LPS, which is excessively produced by intestinal gram-negative bacteria, has a significant effect on inflammation that triggers the accumulation of Aß plaques and tau fibrils in the pathogenesis of AD (11). Although LPS is normally too large to cross the BBB, localized LPS findings with Aβ plaques in AD brains are related to disease-induced BBB permeability in neurological processes (12). These findings support the relationship between the leaky gut barrier and the leaky BBB, which leads to neurological diseases by disrupting brain homeostasis via LPS (13).

Brain-derived neurotrophic factor (BDNF) is a widely distributed neurotrophin in the brain that is involved in many critical processes, from plasticity to neuronal survival and dendritic branching (14). Because of this versatile function, changes in BDNF levels in many neuropathological conditions have been examined in most previous studies to obtain important clues regarding the disease process. Therefore, alterations in BDNF levels may be used as a biomarker for neurological diseases (15). In the current study, we aimed to compare LPS and BDNF levels in the blood plasma of patients with different neurological diseases, such as AD, PD, MS, and epilepsy. In addition, we aimed to contribute to the pathophysiology of neurological diseases by correlating our findings with various biochemical parameters of patients.

MATERIALS AND METHODS

Patients

The study groups consisted of sixty patients (AD, n=15; PD, n=15; MS, n=15; and epilepsy, n=15) and 15 healthy individuals (control group) were recruited from the Department of Neurology in Bezmialem Vakif University Hospital, Istanbul, Turkiye. The study was approved by the Bezmialem Vakif University Research Ethics Committee (07.06.2017, No:12/27; 05/07.2017, No:14/18; 10.07.2020, No:08/117; 10.03.2021, No:3/11) and informed consent was obtained from all participants. In addition, this study was conducted in accordance with the ethical principles of the Declaration of Helsinki. According to the power analysis based on the findings from the post-hoc analysis, assuming a difference of 5.07 between means and a pooled standard deviation of 10, the required sample size at a 95% confidence level was determined to be n1=n2=n3=n4=n5=15, with a total of n=75, achieving a study power of 85%.

Sample Collection

Peripheral blood samples from patients and controls were transported to the laboratory via cold chain transport with EDTA tubes. Blood samples were centrifuged for 10 min at 10,000 g and 4°C to obtain plasma. The obtained plasma was then aliquots and stored at -80°C until further experiments.

Biochemical Measurements

Demographic and clinical characteristics, medication use, and biochemical variables of the patients were obtained from hospital records. Blood sampling for C-reactive protein (CRP) and other biochemical parameters (the levels of creatinine, lactate dehydrogenase (LDH), calcium, potassium, and sodium) was performed using the same blood sample used in the determination of BDNF and LPS levels. CRP was analyzed using a turbidimetric method. Biochemical measurements were performed using standard clinical laboratory methods with certified assays at the Bezmialem Vakif University Hospital clinical laboratory.

Determination of Protein Levels by Enzyme-Linked Immunosorbent Assay

Plasma samples stored at -80^{0}C were taken on ice, and necessary dilutions were performed for optimization of the kits. LPS levels were analyzed using an enzyme-linked immunosorbent assay (ELISA) Kit for Human Lipopolysaccharides (MyBioSource, Inc., San Diego, CA, USA) according to the manufacturer's instructions. BDNF levels were analyzed with Uncoated Human BDNF ELISA Kit (Elabscience, Memorial Drive, Suite 108, Houston, Texas, USA). The LPS and BDNF levels in the last step were determined using absorbance at 450 nm with a Multiskan GO microplate reader (Thermo Fisher Scientific, Boston, MA, USA).

Statistical Analyses

In this study, data distribution was examined using the Shapiro-Wilk test. Comparisons of non-normally distributed data between 5 independent groups were performed using the Kruskal–Wallis (K-W) test. Post hoc comparisons were made using the Dunn test. In all pairwise comparisons, significance values were adjusted using the Bonferroni correction for

multiple tests. Descriptive statistics of numerical data were presented with median (min-max) according to distribution. Categorical data were represented by frequency (percentage). The correlations of relevant biochemical parameters with LPS and BDNF levels were calculated using Pearson's correlation coefficient. All statistical analyses were performed and reported in IBM SPSS Statistics 26.0 programme at 0.05 significance level and 95% confidence level.

Table 1. Demographic and biochemical data of the participants.

	Control	Epilepsy	Alzheimer's Disease	Parkinson's Disease	Multiple Sclerosis	p-value
Gender (Female / Male)	10/5	12/3	6/9	9/6	11/4	0.189#
Age years, mean ± SD	28.40 ± 4.014	33.87 ± 12.438	75.60 ± 6.663	65.73 ± 9.316	37.27 ± 10.333	0.000
Na^+ Mean (Min.-Max.) Reference value 135-145 mmol/L	134.53 (123-143)	136.13 (128-140)	138.27 (133-142)	138.27 (135-143)	139.60 (137-143)	**0.008***
K^+ Mean (Min.-Max.) Reference value 3.5-5.1 mmol/L	6.5733 (3.67-10.00)	5.5193 (3.82-10.00)	5.1320 (3.80-10.00)	4.8653 (3.61-10.00)	4.3347 (3.81-4.72)	0.06
Ca^{++} Mean (Min.-Max.) Reference value 8.3-10.6 mg/dL	6.167 (1.0-10.0)	7.753 (1.0-10.0)	8.080 (1.2-9.8)	8.693 (1.5-10.2)	9.567 (8.8-10.4)	0.117
CRP Mean (Min.-Max.) Reference value 0-5 mg/L	4.6840 (0.20-36.80)	2.5273 (0.02-15.00)	4.7853 (0.02-33.33)	6.0640 (0.12-86.36)	0.9540 (0.19-9.18)	**0.019***
Creatinine Mean (Min.-Max.) Reference value 0.5-1 mg/dL	0.7280 (0.44-1.04)	0.7147 (0.56-1.02)	0.9653 (0.64-1.86)	1.0013 (0.73-3.25)	0.7593 (0.59-1.01)	**0.002***
LDH Mean (Min.-Max.) Reference value 122-222 U/L	164.53 (118-210)	173.47 (135-294)	208.80 (162-285)	174.93 (39-249)	180.53 (126-250)	**0.018***

CRP, C-reactive Protein; LDH, Lactate Dehydrogenase. Kruskal-Wallis Test was used to compare numerical data according to disease groups. Asterisks (*) indicate statistical significance. #Chi-Square test was used to compare

Table 2. Pairwise comparisons of diseases

	Na^+ Adj. Sig.	CRP Adj. Sig.	Creatinine Adj. Sig.	LDH Adj. Sig.
Control -Epilepsy	1.000	1.000	1.000	1.000
Control-Parkinson's Disease	1.000	0.858	1.000	1.000
Control-Alzheimer's Disease	0.613	1.000	0.202	**0.014***
Control-Multiple Sclerosis	**0.009***	1.000	1.000	1.000
Epilepsy-Parkinson's Disease	1.000	1.000	**0.014***	1.000
Epilepsy-Alzheimer's Disease	1.000	0.593	**0.037***	0.067
Epilepsy-Multiple Sclerosis	**0.032***	1.000	1.000	1.000
Parkinson's Disease-Alzheimer's Disease	1.000	**0.010***	1.000	0.370
Parkinson's Disease-Multiple Sclerosis	0.895	1.000	0.226	1.000
Alzheimer's Disease-Multiple Sclerosis	1.000	0.170	0.469	0.443

Significance values have been adjusted by the Bonferroni correction for multiple tests. The asterisk (*) in the table indicates statistical significance, $p<0.05$. Adj. Sig., Adjusted significance; CRP, C-reactive protein; LDH, lactate dehydrogenase.

RESULTS

Clinical Assessments

The study cohort consisted of 60 patients, 15 patients in each disease group (Epilepsy, AD, PD, and MS), and 15 healthy individual in the control group. According to the demographic data, 64% (n=48) of the participants were female and 36% (n=27) of them were male. The average age of all participants was 48.17 years, while the distribution within disease groups ranged from 21 to 85. The demographic data of the participants are presented in Table 1. There was a significant difference in the age of participants ($p<0.001$). Patients with AD and PD were older than the other patients and healthy controls (Table 1).

In addition, certain biochemical parameters were evaluated in the serum of patients and controls. These parameters were the levels of Na^+, K^+, Ca^{++}, Creatinine, CRP, and LDH (Table 1). According to the statistical analysis, a significant difference was found among the groups in terms of Na^+, CRP, creatinine, and LDH values ($p<0.05$). On the other hand, there was no change in the levels of K^+ and Ca^{++} with studied neurological dysfunctions ($p>0.05$).

Compared with the healthy controls, there was an increase in the levels of Na^+ and LDH in patients with neurological disease (Table 1). Post-hoc pairwise comparisons indicated that the increase in the levels of Na^+ was significant in patients with MS ($p=0.009$, Table 2). In addition, MS patients had significantly higher Na^+ levels than that of patients with epilepsy ($p=0.032$). The increase in LDH reached significant values only in the AD patients ($p=0.014$). However, these values were in the normal range.

Compared with the control group, the CRP values were decreased in the epilepsy and MS groups and were increased in the AD and PD groups (Table 1). The CRP level was significantly higher in the PD group than in the AD group, and it was out of the normal range ($p=0.010$). Lastly, the creatinine values were significantly increased in the PD and AD groups compared with the control group ($p=0.020$ and $p=0.009$, respectively). In addition, compared with the epilepsy patients, the creatinine levels were significantly increased in the AD and PD patients ($p=0.037$ $p=0.014$, respectively) (Table 1).

LPS and BDNF

According to the Kruskal-Wallis test, no significant difference was found in LPS levels among the groups. The LPS level was increased, especially in PD patients, compared with the control group (Figure 1). However, there was no change in LPS values among the other neurological disorders.

Although no significant difference was observed between the groups, serum BDNF levels were observed to be higher in the MS group than in the other groups, as shown in Figure 2, but the difference did not reach the expected significance level.

Correlation Analysis of LPS and BDNF Levels According to Biochemical Parameters

Correlations of LPS and BDNF with the levels of Na^+, K^+, Ca^{++}, CRP, creatinine, and LDH were presented in Table 3. Pearson's correlation analysis showed a statistically significant positive correlation between LPS and CRP levels of epilepsy patients ($r=0.753$, $p=0.002$). In patients with AD, LPS levels were negatively correlated with the levels of LDH ($r=-0.521$, $p=0.047$).

Table 3. Pearson's correlation of LPS and BDNF levels with biochemical parameters

	Control (n=15)		Epilepsy (n=15)		Alzheimer's Disease (n=15)		Parkinson's Disease (n=15)		Multiple Sclerosis (n=15)	
LPS correlations with	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
Na^+ (mmol/L)	-0.357	0.191	-0.348	0.223	-0.057	0.839	0.131	0.641	0.166	0.554
K^+ (mmol/L)	0.140	0.618	0.205	0.481	0.120	0.671	0.168	0.549	0.068	0.810
Ca^{++} (mg/dL)	-0.128	0.650	0.161	0.582	-0.344	0.209	-0.171	0.542	0.252	0.366
CRP (mg/L)	0.471	0.077	0.753*	0.002	-0.502	0.056	-0.265	0.339	0.178	0.525
Creatinine (mg/dL)	-0.036	0.899	0.192	0.512	-0.131	0.643	-0.076	0.788	0.179	0.524
LDH (U/L)	0.125	0.657	0.233	0.422	-0.521*	0.047	0.456	0.088	0.232	0.405
BDNF correlations with	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
Na^+ (mmol/L)	-0.185	0.509	-0.052	0.855	-0.498	0.059	0.159	0.571	-0.005	0.985
K^+ (mmol/L)	0.353	0.197	0.494	0.061	-0.136	0.629	0.161	0.566	-0.336	0.221
Ca^{++} (mg/dL)	-0.086	0.760	-0.085	0.763	-0.240	0.388	0.206	0.461	-0.437	0.104
CRP (mg/L)	-0.273	0.325	0.191	0.494	-0.005	0.985	-0.530*	0.042	-0.469	0.078
Creatinine (mg/dL)	0.211	0.450	-0.156	0.578	0.025	0.929	-0.023	0.934	0.063	0.825
LDH (U/L)	-0.149	0.595	-0.216	0.439	-0.172	0.541	0.479	0.071	-0.482	0.069

n is the number of individuals. Statistical evaluation using Pearson's Correlation. LPS, lipopolysaccharide; BDNF, brain-derived neurotrophic factor; CRP, C-reactive protein; LDH, lactate dehydrogenase * $p<0.05$.

Table 4. Receiver operating characteristic (ROC) models for healthy controls vs. patients with epilepsy, Alzheimer's Disease, Parkinson's Disease, and Multiple Sclerosis

	Epilepsy	Alzheimer's Disease	Parkinson Disease	Multiple Sclerosis
BDNF				
AUC	0.60	0.58	0.53	0.63
Standard Error	0.119	0.113	0.112	0.103
95% Confidence Interval	0.410-0.777	0.391-0.760	0.335-0.709	0.429-0.833
p-value	0.38	0.45	0.78	0.22
Sensitivity	86.67	73.33	66.7	66.7
Specificity	60.0	60.0	40.0	60.0
Criterion	<7341.24	<7392.39	<7213.35	<7639.64
LPS				
AUC	0.58	0.67	0.69	0.59
Standard Error	0.110	0.100	0.102	0.107
95% Confidence Interval	0.384-0.760	0.476-0.831	0.495-0.844	0.384-0.803
p-value	0.45	0.08	0.06	0.38
Sensitivity	57.1	80.0	73.3	66.7
Specificity	53.3	46.7	60.0	46.7
Criterion	>564.54	>539.43	>583.76	>577.85

AUC: Area under the ROC Curve; LPS, lipopolysaccharide; BDNF, brain-derived neurotrophic factor

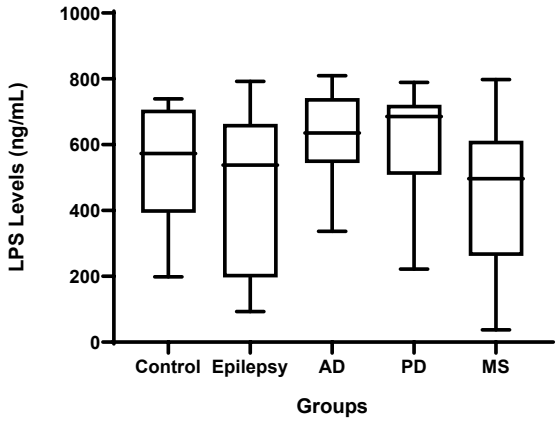

Figure 1. Distribution of LPS (ng/mL) ELISA levels by groups.

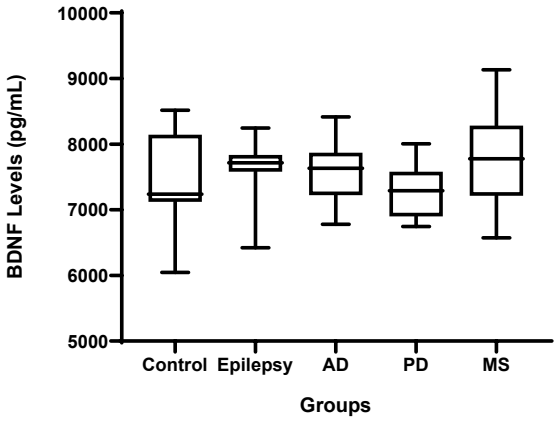

Figure 2. Distribution of BDNF (pg/mL) ELISA levels according to groups.

In addition, there was a statistically significant negative correlation between BDNF and CRP levels (r=0.53, p=0.042) in patients with PD.

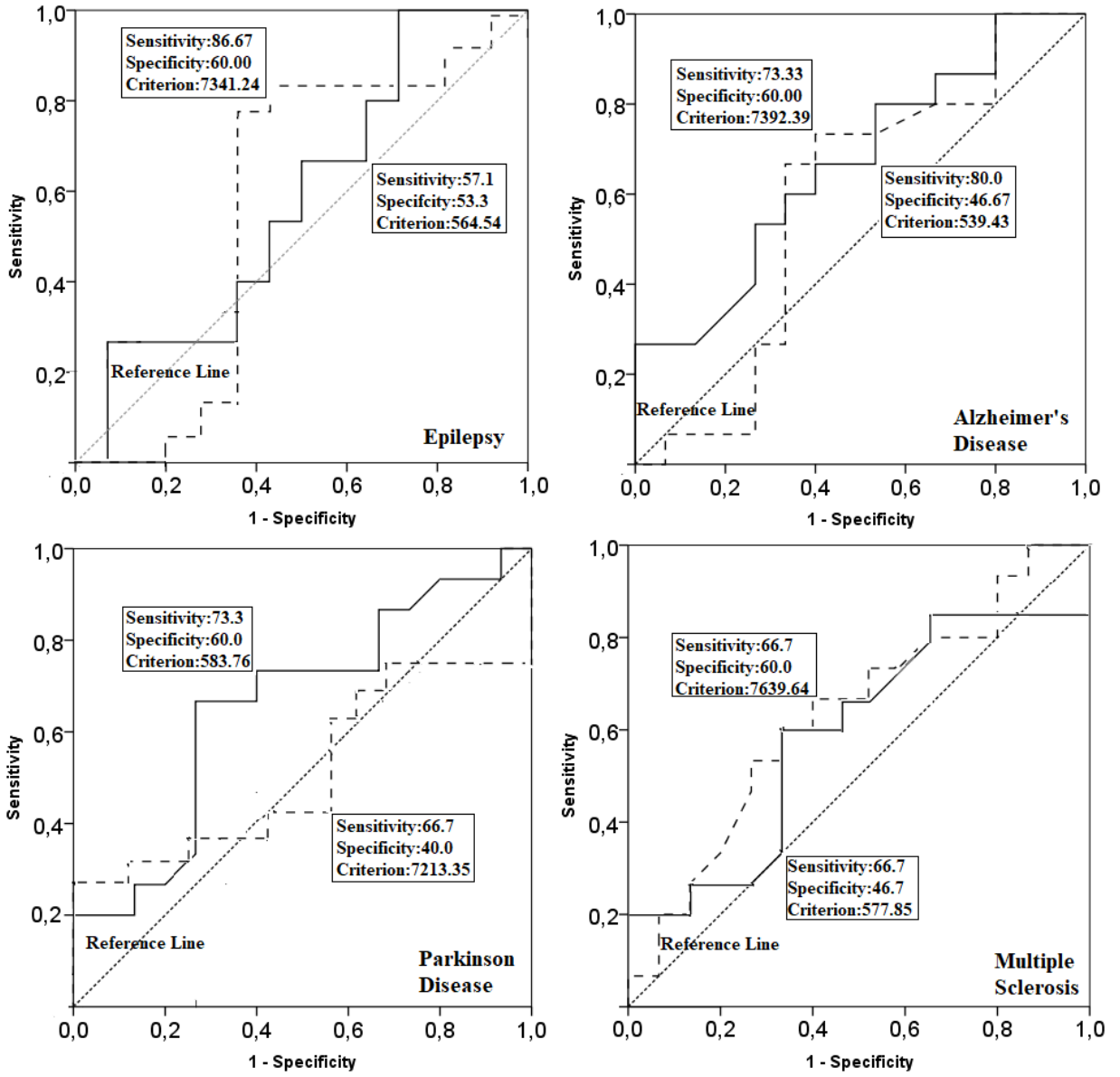

Figure 3. Receiver-operating characteristic (ROC) curves for the levels of BDNF and LPS.

Receiver Operating Characteristic (ROC) Curve Analysis of LPS and BDNF Levels for Differentiating Neurological Diseases

The levels of LPS and BDNF in epilepsy, AD, PD, and MS were analyzed by ROC curve analysis to determine the strength of the levels of LPS and BDNF in blood serum to differentiate distinct neurological diseases (Figure 3, Table 4). According to the ROC curves, the LPS levels had better sensitivity and specificity for distinguishing PD from healthy controls (Table 4). LPS>583.76 pg/mL as the cut-off value, differentiated patients with PD from healthy individuals (sensitivity 73.0%, specificity 60.0%). However, BDNF did not yield an improvement to discriminate in patients with neurological disorders and healthy individuals (Table 4).

DISCUSSION

According to the reports, the global economic and social burden of neurological diseases is increasing daily; one in every three people is affected at some point in life, and deaths due to neurological diseases have increased by 40% in the last 30 years (16, 17). Therefore, in the current study, we aimed to determine the relationships between the levels of BDNF

and LPS in blood plasma and biochemical findings regarding neuroinflammation for the early diagnosis of neurological diseases, including epilepsy, AD, PD, and MS.

In this context, LPS, which causes inflammation and metabolic changes, was examined in selected neurological disorders, and it was found that its levels were significantly increased, especially in PD, rather than in other studied neurological disorders. LPS causes a wide variety of metabolic changes when its levels increase in the blood because of bacterial infections or increased intestinal barrier permeability. An increase in blood LPS triggers the production of pro-inflammatory cytokines and initiates the inflammation process (18). The increase in LPS levels in the blood also manifests endotoxemia and increases the biomarkers of inflammation and infection such as CRP (19). In PD, we observed an increase in CRP levels parallel to an increase in LPS levels, which was also observed in previous studies (20). This outcome was based on the endotoxin hypothesis of PD, which proposes that LPS endotoxins contribute to the pathogenesis of this disorder (21).

Furthermore, we also found a statistically significant positive correlation between LPS and CRP levels in the epilepsy group. In patients with epilepsy, the CRP levels were significantly lower than the control levels. This can be related to the interaction of complement systems with each other. Previous studies have shown that dysregulation of the complement system may be related to epileptogenesis in both animal models and human studies (22). We also found a statistically significant negative relationship between plasma BDNF levels and the CRP trend in the PD group. It is emphasized in studies on PD that the infiltration of peripheral lymphocytes and neutrophils into the brain due to disruption of the integrity of the BBB or damage to the ion transport system may disrupt brain homeostasis and cause ROS production and neuroinflammation (23). The negative correlation between BDNF and CRP levels, which we found to be compatible with all of this literature, indicates that systemic metabolic processes are effective in the pathogenesis of PD and that biomarkers can be detected in serum and plasma samples via metabolomic studies. Studies have shown that CRP levels are high in patients with PD and have been described as a biomarker, particularly in the early stages of PD (24, 25). In addition, the negative relationship between CRP levels and BDNF in our patients with PD was completely consistent with the literature. Studies have shown that serum BDNF levels are significantly reduced in patients undergoing PD and in animal models (26, 27). The protective effects of BDNF are achieved through the activation of the TrkB/MAPK/ERK1/2/IP3K/Akt pathway. Activation of this pathway results in a reduction of apoptosis, as well as neurotoxicity, because molecules are released from apoptotic cells like nitric oxide (NO) (28). PD exhibits elevated levels of oxidative stress, increased NO production, and heightened apoptosis as well (29, 30). In light of these data, the increase in CRP levels, which is a parameter related to inflammation, and the decrease in BDNF, which is a neuroprotective molecule, clearly indicate neuroinflammation-related cell loss. For this reason, additional studies on a larger cohort in the future are warranted, and this negative relationship, especially serum CRP levels, may be a biomarker for the early stage of PD.

Another finding of our study was the statistically significant negative correlation between LPS levels and LDH levels in the AD group. The lactate shuttle is crucial in brain metabolism for long-term memory processes (31). LDH is responsible for the lactate-pyruvate cycle between neurons and glia in several physiological processes, but its function is not fully known (32). However, recent studies have shown that LDH is highly effective against beta-amyloid accumulation and ageing, which are components of neurodegeneration processes (33, 34). In a study conducted in rats, memory loss and learning problems in avoidance behaviour were found when the lactate mechanism in astrocytes was disrupted (35). In our study, we found that the LDH parameter was negatively correlated with LPS serum levels in patients with Alzheimer's disease. The statistically significant increase in LDH levels is consistent with the general literature on neurodegeneration and LDH (36, 37). Although additional studies and a larger cohort are needed to confirm this result, the high LDH level we obtained is associated with impaired energy metabolism in the brain, as shown in many studies related to neurodegenerative diseases and even depression (38-40).

According to the ROC analysis results, LPS levels demonstrated notable sensitivity and specificity for distinguishing Parkinson's disease from healthy controls, with a determined cut-off value of 583.76 pg/mL, achieving a sensitivity of 73.0% and specificity of 60.0%. Conversely, BDNF did not exhibit significant discriminatory power between patients with neurological disorders and healthy subjects. These findings underscore the potential of LPS as an endotoxin, in non-invasively discerning PD characterized by neurodegeneration. We propose that obtaining statistically significant results on BDNF levels could be feasible with a larger cohort.

In our study, no statistically significant differences were found between the serum LPS and BDNF levels and blood biochemical parameters in the MS group. We believe that the drugs used by patients may mask the statistical significance and affect blood parameters. We also plan to examine MS, which is a highly heterogeneous neurological disease, in a larger cohort with additional validation in future experiments.

CONCLUSION

In conclusion, our study sheds light on the intricate relationships between biomarkers and biochemical parameters in various neurological diseases, including epilepsy, AD, PD, and MS. We found significant associations between blood LPS, CRP, BDNF, and LDH levels with different neurological conditions. Specifically, our finding, supported by the positive correlation between CRP and LPS levels, suggests that CRP is an indicator of neurodegeneration in epilepsy. Additionally, although the difference between the groups was not significant, LPS levels were found to be higher in the PD group than in the

control. Moreover, the negative correlation between BDNF and CRP levels in PD patients underscores the importance of neuroprotective mechanisms in combating inflammation-associated cell loss. Additionally, the negative correlation between LPS and LDH levels in AD patients with high LDH levels suggests disrupted energy metabolism, a common feature of neurodegenerative processes. Although our study did not reveal significant differences in serum LPS and BDNF levels among patients with MS, we recognize the need for further investigation with larger cohorts to better understand the complexities of this heterogeneous disease. Overall, our findings suggest the potential utility of these biomarkers in the early diagnosis and monitoring of neurological diseases, offering avenues for future research aimed at improving patient outcomes and developing therapeutic strategies.

Ethics Committee Approval: The study was approved by the Bezmialem Vakif University Research Ethics Committee (07.06.2017, No:12/27; 05/07.2017, No:14/18; 10.07.2020, No:08/117; 10.03.2021, No:3/11).

Informed Consent: Informed written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- N.D.K., B.E., E.Y.; Data Acquisition: N.D.K., B.E., S.S., C.T., A.B., F.U., G.B., A.E.G., F.A., B.S., M.Y., Z.E.; Data Analysis/Interpretation: B.E., N.D.K.; A.Y.T.; Drafting Manuscript- N.D.K., B.E.; Critical Revision of Manuscript- N.D.K., B.E.; Final Approval and Accountability- B.E.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: The authors declare that this study has received no financial support.

REFERENCES

1. Riessland M. Is cellular senescence of dopaminergic neurons the cause of local inflammation in the midbrain observed in Parkinson's Disease? J Cell Immun 2020; 2(5): 201-4.
2. Sweeney MD, Kisler K, Montagne A, Toga AW, Zlokovic BV. The role of brain vasculature in neurodegenerative disorders. Nat Neurosci 2018; 21(10): 1318-31.
3. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 2017; 541(7638): 481-7.
4. Ballabh P, Braun A, Nedergaard M. The blood–brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis 2004; 16(1): 1-13.
5. Bowman GL, Kaye JA, Moore M, Waichunas D, Carlson NE, Quinn JF. Blood-brain barrier impairment in Alzheimer disease: stability and functional significance. Neurology 2007; 68(21): 1809-14.
6. Gray MT, Woulfe JM. Striatal blood-brain barrier permeability in Parkinson's disease. J Cereb Blood Flow Metab 2015; 35(5): 747-50.
7. Waubant E. Biomarkers indicative of blood-brain barrier disruption in multiple sclerosis. Dis Markers 2006; 22(4): 235-44.
8. Gorter JA, Aronica E, van Vliet EA. The roof is leaking and a storm is raging: repairing the blood-brain barrier in the fight against epilepsy. Epilepsy Curr 2019; 19(3): 177-81.
9. Needham BD, Trent MS. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 2013; 11(7): 467-81.
10. Raetz CR, Whitfield C. Lipopolysaccharide endotoxins. Annu Rev Biochem 2002; 71: 635-700.
11. Brown GC. The endotoxin hypothesis of neurodegeneration. J Neuroinflammation 2019; 16(1): 180.
12. Zhan X, Stamova B, Sharp FR. Lipopolysaccharide associates with amyloid plaques, neurons and oligodendrocytes in Alzheimer's Disease brain: a review. Front Aging Neurosci 2018; 10: 42.
13. Sochocka M, Donskow-Lysoniewska K, Diniz BS, Kurpas D, Brzozowska E, Leszek J. The gut microbiome alterations and inflammation-driven pathogenesis of Alzheimer's Disease-a critical review. Mol Neurobiol 2019; 56(3): 1841-51.
14. Draper B, Yee WL, Pedrana A, Kyi KP, Qureshi H, Htay H, et al. Reducing liver disease-related deaths in the Asia-Pacific: the important role of decentralised and non-specialist led hepatitis C treatment for cirrhotic patients. Lancet Reg Health West Pac 2022; 20: 100359.
15. Lima Giacobbo B, Doorduin J, Klein HC, Dierckx RA, Bromberg E, de Vries EF. Brain-derived neurotrophic factor in brain disorders: focus on neuroinflammation. Mol Neurobiol 2019; 56: 3295-312.
16. Olesen J, Gustavsson A, Svensson M, Wittchen HU, Jönsson B, Group CS, et al. The economic cost of brain disorders in Europe. Eur J JNeurol 2012; 19(1): 155-62.
17. Feigin VL, Nichols E, Alam T, Bannick MS, Beghi E, Blake N, et al. Global, regional, and national burden of neurological disorders, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurol 2019; 18(5): 459-80.
18. Rhee SH. Lipopolysaccharide: basic biochemistry, intracellular signaling, and physiological impacts in the gut. Intest Res 2014; 12(2): 90-5.
19. Marsik C, Sunder-Plassmann R, Jilma B, Kovar FM, Mannhalter C, Wagner O, et al. The C-reactive protein (+)1444C/T alteration modulates the inflammation and coagulation response in human endotoxemia. Clin Chem 2006; 52(10): 1952-7.
20. Wight RD, Tull CA, Deel MW, Stroope BL, Eubanks AG, Chavis JA, et al. Resveratrol effects on astrocyte function: relevance to neurodegenerative diseases. Biochem Biophys Res Commun 2012; 426(1): 112-5.
21. Brown GC, Camacho M, Williams-Gray CH. The endotoxin hypothesis of Parkinson's disease. Mov Disord 2023; 38(7): 1143-55.
22. Liguori C, Romigi A, Izzi F, Placidi F, Nuccetelli M, Cordella A, et al. Complement system dysregulation in patients affected by idiopathic generalized epilepsy and the effect of antiepileptic treatment. Epilepsy Res 2017; 137: 107-11.
23. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. Nat Med 2013; 19(12): 1584-96.
24. Akil E, Bulut A, Kaplan I, Ozdemir HH, Arslan D, Aluclu MU. The increase of carcinoembryonic antigen (CEA), high-sensitivity C-reactive protein, and neutrophil/lymphocyte ratio in Parkinson's disease. Neurol Sci 2015; 36: 423-8.
25. Kim R, Kim H-J, Kim A, Jang M, Kim A, Kim Y, et al. Peripheral blood inflammatory markers in early Parkinson's disease. J Clin Neurosci 2018; 58: 30-3.

26. Baquet ZC, Bickford PC, Jones KR. Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta. J Neurosci 2005; 25(26): 6251-9.
27. Parain K, Murer MG, Yan Q, Faucheux B, Agid Y, Hirsch E, et al. Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra. Neuroreport 1999; 10(3): 557-61.
28. Numakawa T, Suzuki S, Kumamaru E, Adachi N, Richards M, Kunugi H. BDNF function and intracellular signaling in neurons. Histol Histopathol 2010; 25(2): 237-58.
29. Kaur R, Mehan S, Singh S. Understanding multifactorial architecture of Parkinson's disease: pathophysiology to management. Neurol Sci 2019; 40: 13-23.
30. Park H, Kang S, Nam E, Suh Y-H, Chang K-A. The protective effects of PSM-04 against beta amyloid-induced neurotoxicity in primary cortical neurons and an animal model of Alzheimer's disease. Front Pharmacol 2019; 10: 2.
31. Ivanov A, Mukhtarov M, Bregestovski P, Zilberter Y. Lactate effectively covers energy demands during neuronal network activity in neonatal hippocampal slices. Front Neuroenergetics 2011; 3: 2.
32. Dienel GA. Brain lactate metabolism: the discoveries and the controversies. J Cereb Blood Flow Metab 2012; 32(7): 1107-38.
33. Niccoli T, Kerr F, Snoeren I, Fabian D, Aleyakpo B, Ivanov D, et al. Activating transcription factor 4-dependent lactate dehydrogenase activation as a protective response to amyloid beta toxicity. Brain Commun 2021; 3(2): fcab053.
34. Long DM, Frame AK, Reardon PN, Cumming RC, Hendrix DA, Kretzschmar D, et al. Lactate dehydrogenase expression modulates longevity and neurodegeneration in Drosophila melanogaster. Aging (Albany NY) 2020; 12(11): 10041.
35. Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, et al. Astrocyte-neuron lactate transport is required for long-term memory formation. Cell 2011; 144(5): 810-23.
36. Adeva-Andany M, López-Ojén M, Funcasta-Calderón R, Ameneiros-Rodríguez E, Donapetry-García C, Vila-Altesor M, et al. Comprehensive review on lactate metabolism in human health. Mitochondrion 2014; 17: 76-100.
37. Kreisberg RA. Lactate homeostasis and lactic acidosis. Ann Intern Med 1980; 92(2 Part 1): 227-37.
38. Dong S-Y, Guo Y-J, Feng Y, Cui X-X, Kuo S-H, Liu T, et al. The epigenetic regulation of HIF-1α by SIRT1 in MPP+ treated SH-SY5Y cells. Biochem Biophys Res Commun 2016; 470(2): 453-9.
39. Yao Q, Liu H, Li Y. Low levels of serum LDH are associated with depression and suicide attempts. Gen Hosp Psychiatry 2022; 79: 42-9.
40. Doktorchik C, Patten S, Eastwood C, Peng M, Chen G, Beck CA, et al. Validation of a case definition for depression in administrative data against primary chart data as a reference standard. BMC Psychiatry 2019; 19:1-8.

Experimed 2024; 14(2): 102-109

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1479944

Effect of *Lemon Verbena* Polyphenol on Glycerol Channel Aquaporin 7 Expression in 3T3-L1 Adipocytes

Orkide Palabiyik[1], Emine Kilic-Toprak[2], Deniz Sumnulu[3], Julide Tozkir[1], Aysegul Cort[4]

[1]Health Services Vocational College, Trakya University, Edirne, Turkiye
[2]Department of Physiology, Faculty of Medicine, Pamukkale University, Denizli, Turkiye
[3]Technology Research Development Application and Research Centre, Trakya University, Edirne, Turkiye
[4]Department of Biochemistry, Faculty of Medicine, Pamukkale University, Denizli, Turkiye

ORCID ID: O.P. 0000-0002-3488-3740; E.K.T. 0000-0002-8795-0185; D.S. 0009-0009-0693-3569; J.T. 0000-0002-8436-8309; A.C. 0000-0001-8946-7173

Cite this article as: Palabiyik O, Kilic-Toprak E, Sumnulu D, Tozkir J, Cort A. Effect of *Lemon Verbena* polyphenol on glycerol channel aquaporin 7 expression in 3T3-L1 adipocytes. Experimed 2024; 14(2): 102-109.

ABSTRACT

Objective: Polyphenols are of great interest in obesity prevention approaches. The aquaglyceroporin 7 (*AQP7*) channel is involved in the transport of glycerol across cell membranes in adipose tissue. This study aimed to explore how *lemon verbena* (LV) polyphenols affect the expression of the glycerol channels *AQP7* and *perilipin 1* (*PLIN1*) in 3T3-L1 hypertrophic adipocytes.

Materials and Methods: Hypertrophic adipocyte cells (H) were treated with LV at two different doses of 200 µg/mL (H-LV200) and 400 µg/mL (H-LV400). In addition, 0.1 µM β3-AR agonist (CL316243) and 0.1 µM β3-AR antagonist (L7483337) were applied to the cells at both doses. *AQP7* and *PLIN1* gene expressions were determined by real time-polymerase chain reaction (RT-PCR), and glycerol levels were determined by enzyme-linked immunosorbent assay (ELISA).

Results: Hypertrophic adipocytes showed increased *AQP7* and *PLIN1* gene expression and glycerol content compared with the control group. H-LV200 and CL316243 treatment together increased *AQP7* gene expression, whereas H-LV400 and L7483337 treatment together decreased *AQP7* gene expression.

Conclusion: The data indicated that both doses of LV inhibited glycerol production by suppressing *AQP7* and *PLIN1* gene expression. Approaches to regulate *AQP7* gene expression in adipose tissue using plant-derived polyphonic compounds are considered a healthy and innovative approach to combat and manage metabolic diseases, including obesity.

Keywords: Polyphenol, adipocyte, aquaglyceroporin, glycerol, obesity

INTRODUCTION

Obesity is a global public health problem that leads to chronic diseases, such as diabetes, cardiovascular disease, and cancer (1). In obesity, adipose tissue homeostasis is disturbed, leading to the proliferation and enlargement of adipocytes (2). Currently, bioactive compounds derived from natural foods are attracting considerable interest as potential strategies to combat obesity. Among these compounds, polyphenols, which are abundant in fruits, vegetables, cereals, and beverages, stand out. In fact, specific plant-derived polyphenols are increasingly being considered viable alternatives to obesity (3-5). Numerous polyphenols play an important role in the regulation of essential cellular processes, including growth, differentiation, energy balance, and metabolic homeostasis. These compounds contribute to metabolic health by modulating molecular signalling pathways (6). *Lemon verbena* (LV) is a notable example of these polyphenols. One of its major constituents, verbascoside, a phenylpropanoid, plays a crucial role in imparting the plant's antioxidant and anti-inflammatory properties. Polyphenolic extracts from LV have been shown

Corresponding Author: Orkide Palabiyik **E-mail:** orkidepalabiyik@trakya.edu.tr
Submitted: 07.05.2024 **Revision Requested:** 27.06.2024 **Last Revision Received:** 16.07.2024 **Accepted:** 24.07.2024

to suppress intracellular lipid accumulation, oxidative stress due to high glucose levels, and inflammation in adipose tissue by acting on various metabolic pathways. Research has potential applications in the prevention and treatment of metabolic diseases (7, 8).

LV extract has been reported to reduce high-glucose-induced metabolic stress in hypertrophic adipocytes and hyperlipidaemia mice through 5'-adenosine monophosphate-activating protein kinase (AMPK)-dependent mechanisms (11). LV has been shown to exert this effect by activating adiponectin through a transcriptional peroxisome proliferation activating receptor gamma (PPAR-γ)-dependent mechanism that correlates with AMPK activation. Through this pathway, LV oxidation increases fatty acid oxidation, suppresses lipogenesis in obesity, and alleviates obesity-related disorders (9).

Aquaporins (AQP) are channel proteins that enable the passage of water and small molecules. In mammals, 13 forms have been identified and classified into subgroups. Recently, the effects of AQPs on adipose tissue homeostasis have been clarified, providing a new perspective in this field. Research indicates that AQPs could serve as targets for drug development aimed at treating diseases related to adipose tissue function and/or dysfunction (10-14). The function of *AQP7*, an aquaglyceroporin, a subgroup of AQPs, primarily stems from its role in enabling glycerol transport across cell membranes (15). Therefore, it plays an important role in regulating glycerol transport from adipose tissue to maintain lipid and energy balance. Under lipogenic conditions of increased insulin stimulation, such as feeding, *AQP7* co-localises with the protein *perilipin 1* (*PLIN1*) at the lipid droplet interface, and the movement of AQP7 to the plasma membrane is inhibited by *PLIN1*. Under lipolytic conditions, such as fasting and exercise, when the energy demand increases, triacylglycerol (TAG) in adipose tissue is hydrolysed, releasing free fatty acids (FFA) and glycerol. Protein kinase A (PKA), which is activated by signalling under these metabolic conditions, weakens the association between *AQP7* and *PLIN1*. The inhibitory effect of *PLIN1* on *AQP7* is removed. *AQP7* translocates to the plasma membrane to release glycerol into the circulation (16). Glycerol is an important organic molecule in carbohydrate and lipid metabolism because of its role in determining plasma glucose levels (17-19). No studies have been identified in the existing literature that investigate the impact of LV plant extract on the expression of the glycerol channel *AQP7* and associated *PLIN1* in 3T3-L1 cells. There are only a few studies on the effects of plant-derived polyphenol applications on *AQP7* and *PLIN1* gene expression (19, 20). Among these studies, no studies have investigated the effects of LV therapy. In our study, we sought to explore the effects of LV polyphenols on *AQP7* and *PLIN1* expression in mature and hypertrophic adipocytes.

MATERIALS AND METHODS

Chemicals and Reagents

In order to investigate into the impact of LV polyphenols on lipid and glucose metabolism, LV polyphenolic extract (27% verbascoside, dry weight) was kindly provided by Dr. María Herranz-López (University Miguel Hernandez, Elche, Spain) (9). The LV aqueous extract contained iridoid glycosides (% dry weight w/w; 2.5%), phenylpropanoid (w/w; 33.5%), and flavonoids (w/w; 4.8%). The total w/w of these identified compounds was 40.8%. Verbascoside comprises 27% phenylpropanoids. LVs were freshly prepared before use and filtered after thawing in cell culture medium. LV polyphenol doses of 200 µg/mL (mature) and 400 µg/mL (hypertrophic adipocytes) were selected and applied to the cells as previously described (9, 21). Under conditions that promote fat breakdown, sympathetic nerves become active, resulting in an increase in catecholamine levels and stimulation of adrenergic receptors in adipose tissue. The β3-AR receptor agonist CL316243 (CL; Cat. No:138908-40-4, Cayman Chemical, Michigan, USA) was used to stimulate fat breakdown in adipose tissue, while the β3-AR antagonist L7483337 (L; Cat. No: 244192-94-7, Tocris Bioscience-Bristol, UK) was used to obstruct its stimulation.

Cell Culture

The preadipocyte cell line 3T3-L1 (American Type Culture Collection, Manassas, VA, USA, CL173™) were cultured in low-glucose (1 g/L) DMEM supplemented with 10% calf serum, 100 g/mL streptomycin, and 100 U/mL penicillin, known as complete medium. The cells were cultured in flasks, with the complete medium being replaced every 2-3 days, and incubated at 37°C in a humidified atmosphere (5% CO_2, 95% air).

Differentiation of the 3T3-L1 Cell Line

Differentiation of 3T3-L1 preadipocyte was performed according to standard protocols (22). Initially, preadipocyte were seeded in 6-well plates using complete medium and cultured until they reached confluence. Next, differentiation was induced by incubating with differentiation medium I (DMI), which contains high-glucose DMEM (4.5 g/L) supplemented with 10% FBS and adipogenic reagents such as 10 µg/mL insulin, 1 µM DXMT, and 0.5 mM IBMX. This DMI was left for 48 h and then changed to differentiation medium II (DMII), which contains insulin and 10% FBS in high-glucose DMEM (4.5 g/L). The DMII was changed every 2-3 days for 10-12 days, obtaining mature adipocytes. After obtaining mature adipocytes, The cells underwent 24-h treatment with LV (200 µg/mL, 400 µg/mL), L (0,1 µM), CL (0.1 µM), LV (200 µg/mL)+L(0.1 µM), LV (400 µg/mL)+L (0.1 µM), LV (200 µg/mL)+CL (0.1 µM), and LV (400 µg/mL)+CL 0.1 µM). Before cell treatment, all extracts were dissolved in either medium or DMSO-enhanced medium and subsequently filtered for sterilisation.

Hypertrophic Adipocyte Model

Once the differentiation to mature adipocytes has occurred, the hypertrophic state of adipocytes can be obtained to simulate obesity-induced adipose tissue via prolonged incubation under high-glucose conditions. To obtain hypertrophic adipocytes from mature adipocytes, the cells were maintained with DMII for at least an additional 7 days. Nearly 20 days

after incubation, a hypertrophic adipocyte model has been observed. It is a well-established model of insulin resistance in adipocytes exposed to metabolic stress (23). After obtaining hypertrophic adipocytes, the cells were treated for 48 h with LV (200 µg/mL, 400 µg/mL), L (0.1 µM), CL (0.1 µM), LV (200 µg/mL)+L (0.1 µM), LV (400 µg/mL)+L (0.1 µM), LV (200 µg/mL)+L (0.1 µM), and LV (400 µg/mL)+CL (0.1 µM). Each extract was dissolved in medium or DMSO-supplemented medium, sterilised by filtration, and then administered to the cells. The planned study groups are as follows;

1. Mature adipocytes without any treatment (control (K))
2. Untreated hypertrophic adipocyte (hypertrophic (H))
3. Hypertrophic adipocytes treated with LV 200 µg/mL alone (H-LV200)
4. Hypertrophic adipocytes treated with LV 400 µg/mL alone (H-LV400)
5. Hypertrophic adipocytes treated with CL alone (H-CL)
6. Hypertrophic adipocytes treated with L alone (H-L)
7. Hypertrophic adipocytes treated with a combination of 200 µg/mL LV and CL (H-LV200+CL)
8. Hypertrophic adipocytes treated with a combination of 400 µg/mL LV and CL (H-LV400+CL)
9. Hypertrophic adipocytes treated with 200 µg/mL combination of LV and L (H-LV200+L)
10. Hypertrophic adipocytes treated with 400 µg/mL combination of LV and L (H-LV400+L)

Cell Viability

To assess the percentage of viable 3T3-L1 cells, approximately 2×10^4 cells per well were seeded in a 96-well plate and treated with the indicated agents. Different incubation protocols were applied to cells, followed by incubation for 24 and 48 h. Cell viability was assessed using the WST-8 Cell Proliferation Kit-8 (Elabscience), following the manufacturer's instructions for proliferation assays. WST-8 reagent was added to each well for 4 h. Upon completion of the assay, the absorbance for each sample was recorded at 450 nm using a microplate reader (BioTec-USA) (24).

Oil-Red-O Staining

To test the suitability of the cell culture model created in our experiments was done in 2 stages. A staining kit (Oil-red-O staining kit, K580-24, Biovision, USA) was used. Cells were seeded in 6-well plates at a density of 100,000 cells/well, as previously described. Following the steps described in the adipocyte differentiation protocol, Oil red O staining was performed at different stages of the cells, and intracellular lipid accumulation was evaluated.

Total RNA Isolation and Gene Expression

Mature cells treated with LV, CL, and L for 24 h, along with hypertrophic adipocytes treated for 48 h, were collected by centrifugation at 1,000 g for 10 min. The cell pellets were lysed using 100 µL of cold lysis buffer. Following a 10-min incubation on ice, the lysates were centrifuged at 14,000 g for 1 min to separate the supernatants. After centrifugation, the supernatants were decanted into fresh microcentrifuge tubes. Mature and hypertrophic cells were harvested using Trizol™ (Cat.No:15596018, Ambion, Invitrogen, California, USA). To determine *AQP7* and *PLIN1* gene expression in LV polyphenol-treated cells, cells were removed from the flask and centrifuged at 1500 rpm for 5 min. Following removal of the medium, total RNA was extracted from the pellets using Trizol™ (Cat.No:15596018, Ambion, Invitrogen, California, USA) reagent, according to the manufacturer's instructions. The quality and quantity of the isolated RNAs were assessed using a Nanodrop ND-2000c (Thermo Scientific-USA). Subsequently, mRNAs were converted into cDNA using a cDNA Reverse Transcription kit (A.B.T.™ Laboratory Industry, Ankara, Turkiye). cDNA-converted samples were stored at -20°C until used in real time-polymerase chain reaction (RT-PCR) analyses. The effects of LV administration to 3T3-L1 hypertrophic adipocyte cells on *AQP7* (F: 5'-TATGGTGCGAGAGTTTCTGG -3', R: 5'-GCCTAGTGCACAATTGGTGA -3') and *PLIN1* (F: 5'-ACAGAGAATATGCCGCCAA-3', R: 5'-GGCTGACTCCTTGTCTGGTG -3') gene expressions were analyzed by RT-PCR. For normalisation of the data obtained because of RT-PCR, glyceraldehyde-3-phosphate-dehidrogenase (GAPDH) (F: 5'-CCTGCCAAGTATGACATCAA -3', R: 5'- AGCCCAGGATGC CCTTTAGT-3') reference gene was used for normalization. We obtained the analysis results from three replicates and normalized the data by calculating the $2^{-\Delta\Delta CT}$ value.

Measurement by ELISA

Glycerol levels in cell medium collected and stored at -80°C were measured using an enzyme-linked-immunosorbent assay (ELISA) kit (BioVision Cat. No: K630-100, CA, USA) according to the manufacturer's instructions (Bio Tek Instruments, Inc, USA).

Statistical Analyses

Results are presented as mean ± standard deviation. The normality of data distribution was assessed using the Shapiro-Wilk test. For comparisons between groups, the Kruskal-Wallis Test and the Mann–Whitney U test were used. A value of $p<0.05$ was considered statistically significant. In this study, the SPSS 20.0 package was used for the statistical analysis of the data.

RESULTS

Association Between *AQP7*/*PLIN1* Gene Expression and Glycerol Concentration in Hypertrophic Adipocytes

A comparison was made between *AQP7* and *PLIN1* gene expression and glycerol concentrations in hypertrophic and non-hypertrophic (mature) control adipocytes. The results indicated that *AQP7* and *PLIN1* gene expression was elevated in hypertrophic adipocytes relative to the control group (Figure 1,

2 and 3). Although the increase in *AQP7* gene expression was not statistically significant (p=0.704), the increase in *PLIN1* gene expression was statistically significant (p<0.001). The glycerol concentration in the medium surrounding hypertrophic adipocytes was significantly elevated compared with that in the medium surrounding mature control group adipocytes (K; 11.77 ± 0.40 ng/mL vs H; 43.79 ± 0.59 ng/mL, p=0.014; Figure 4). Therefore, we inferred that *AQP7*, which is modulated by *PLIN1* in hypertrophic adipocytes, acts as a mediator of glycerol extrusion.

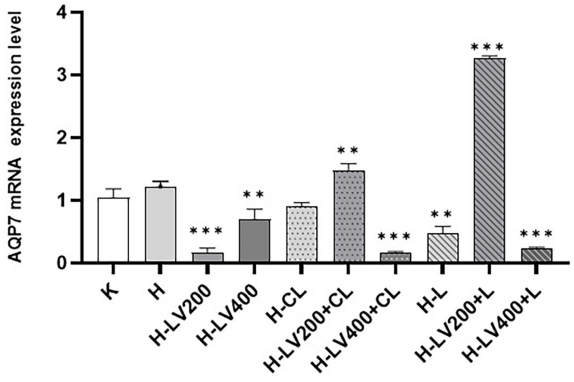

Figure 1. Changes in *AQP7* gene expression on application of 200 and 400 ng/mL doses of LV, the β3-AR receptor agonist-CL and the β3-AR receptor antagonist-L.
K, control; H, hypertrophic adipocyte; LV, *lemon verbena*; CL, CL316243 (β3-AR receptor agonist); L, L7483337 (β3-AR antagonist); **p<0.001; ***p<0.0001.

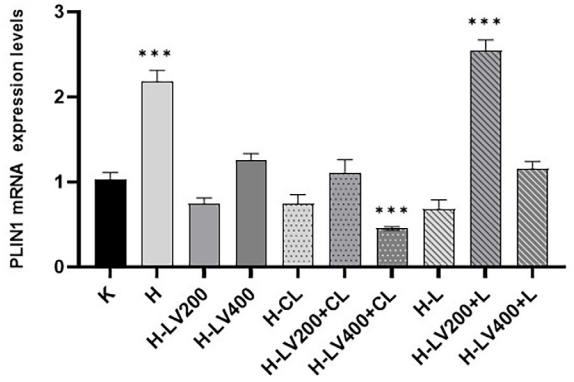

Figure 2. Changes in *PLIN1* gene expression on application of 200 and 400 ng/mL doses of LV, the β3-AR receptor agonist-CL and the β3-AR receptor antagonist-L.
K, control; H, hypertrophic adipocyte; LV, *lemon verbena*; CL, CL316243 (β3-AR receptor agonist); L, L7483337 (β3-AR antagonist); **p<0.0001.

Application of LV at Different Concentrations

When LV polyphenol was administered to hypertrophic adipocytes (H) at doses of 200 and 400 µg/mL, it resulted in the inhibition of *AQP7* gene expression compared with hypertrophic adipocytes (H vs H-LV200 p<0.0001 and H vs H-LV400 p=0.005, respectively; Figure 1). Although gene expression of *PLIN1* was significantly decreased at a dose of 200 µg/mL, there was no statistically significant difference at both doses (H vs H-LV200 p=0.279 and H vs H-LV400 p=0.611, respectively; Figure 2). The glycerol content in the medium of LV-treated cells was decreased (H; 43.79 ± 0.59 ng/mL vs H-LV200; 32.05 ± 1.59 ng/mL, p=0.009; H; 43.79 ± 0.59 ng/mL vs H-LV400; 36.83 ± 0.73 ng/mL, p=0.009; Figure 4). These findings indicate that both LV doses diminished glycerol efflux by suppressing *AQP7* and *PLIN1* gene expression.

Administration of the β3-AR Receptor Agonist CL

The β3-AR receptor agonist CL, which is known to induce anti-obesity effects, did not affect the gene expression of *AQP7* and *PLIN1* when applied alone to hypertrophic cells. This was when compared with the non-hypertrophic mature adipocyte control (K), and the results were non-significant (For *AQP7*, K vs H-CL p=0.846 and for *PLIN1*, K vs H-CL p=0.272, respectively) (Figure 1 and 2). However, the amount of glycerol in the medium of CL-treated hypertrophic adipocytes was increased compared with non-hypertrophic mature adipocytes and hypertrophic control adipocytes. The increase observed in the hypertrophic control group failed to reach statistical significance (K: 11.77 ± 0.40 ng/mL vs H-CL: 62.81 ± 3.63 ng/mL, p<0.0001; H: 43.79 ± 0.59 ng/mL vs H-CL: 62.81 ± 3.63 ng/mL, p=0.098; Figure 4).

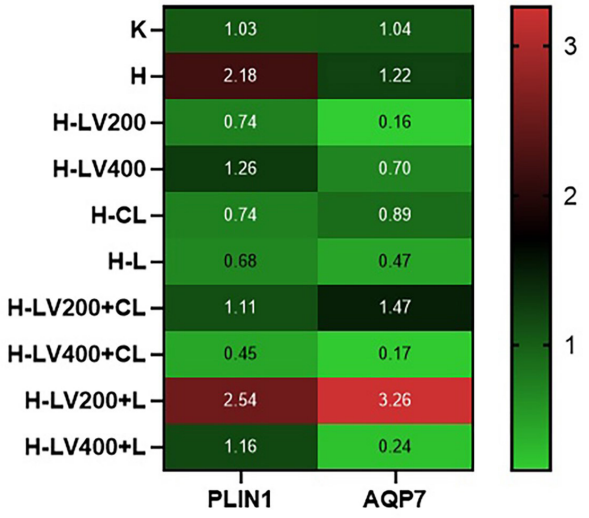

Figure 3. Whole cell *AQP7* and *PLIN1* gene expression levels in the groups are shown together.
K, control; H, hypertrophic adipocyte; LV, *lemon verbena*; CL, CL316243 (β3-AR receptor agonist); L, L7483337 (β3-AR antagonist); **p<0.001; ***p<0.0001.

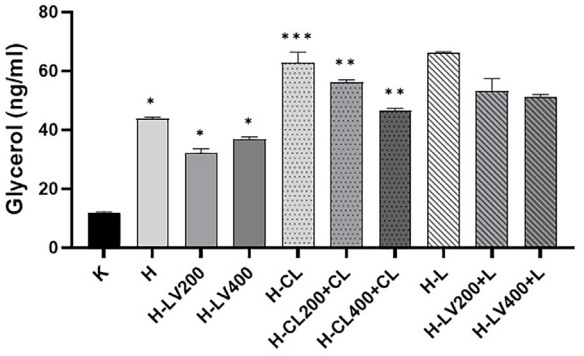

Figure 4. Change of glycerol content in the adipocytes medium when 200 and 400 ng/ml doses of LV, the β3-AR receptor agonist-CL and the β3-AR receptor antagonist-L. K, control; H, hypertrophic adipocyte; LV, *lemon verbena*; CL, CL316243 (β3-AR receptor agonist); L, L7483337 (β3-AR antagonist); *p<0.05; **p<0.001; ***p<0.0001.

When a 200 µg/mL dosage of LV and CL was applied to hypertrophic adipocytes, an increase in *AQP7* gene expression was observed (p=0.005), but no changes in *PLIN1* gene expression were noted (p=0.999).

An analysis of glycerol content showed that both non-hypertrophic mature adipocytes and hypertrophic adipocytes exhibited increased glycerol content compared with the controls. However, although there was an increase in glycerol content in hypertrophic adipocytes compared with the control, it did not reveal any statistical significance (K; 11.77 ± 0.40 ng/mL vs H-LV200+CL; 56.20 ± 0.67 ng/mL, p<0.0001, H; 43.79 ± 0.59 ng/mL vs H-LV200 + CL; 56.20 ± 0.67 ng/mL, p=0.226). When 400 µg/mL dose of LV and CL was applied to hypertrophic adipocytes, *AQP7* and *PLIN1* gene expressions were significantly decreased compared with the non-hypertrophic mature adipocyte control (p<0.0001, p<0.0001, respectively) (Figure 1 and 2). When compared in terms of glycerol concentration released in the media of the cells, glycerol was found to be increased in the LV-treated groups (K; 11.77 ± 0.40 ng/mL vs H-LV400 + CL; 46.35 ± 0.45 ng/mL p=0.005). When hypertrophic adipocytes were compared with the control group, the glycerol content was similar (H; 43.79 ± 0.59 ng/mL vs H-LV400 + CL; 46.35 ± 0.45 ng/mL p=0.729; Figure 4). When CL was co-administered with 200 µg/mL dose of LV, the amount of glycerol increased due to an increase in *AQP7* gene expression. This indicates that CL has different effects on *AQP7* gene expression and glycerol output depending on the LV application dose.

β3-AR Receptor Antagonist L and LV Application at Different Concentrations

AQP7 and *PLIN1* gene expression levels were assessed following the administration of the β3-AR antagonist, L, alone and in combination with two different concentrations of LV. L treatment decreased the gene expression of both *AQP7* and *PLIN1* in hypertrophic adipocytes compared with non-hypertrophic mature adipocytes. Notably, a statistically significant decrease in *AQP7* expression was observed (p<0.0001) and *PLIN1* gene expression was not significant (p<0.079). Treatment with L and LV at a concentration of 200 µg/mL resulted in a noteworthy increase in the gene expression of *AQP7* and *PLIN1* (p<0.0001 vs p<0.0001), in comparison to treatment with L alone. Conversely, treatment with L and LV at a concentration of 400 µg/mL led to a significant decrease in *AQP7* gene expression (p<0.0001; Figure 1). The experimental conditions did not affect *PLIN1* gene expression (p=0.991; Figure 2). When comparing glycerol content, it was found that the administration of L along with LV doses of 200 µg/mL and 400 µg/mL led to a significant increase in comparison with the non-hypertrophic mature adipocyte control (K; 11.77 ± 0.40 ng/mL vs H-LV200+L; 53.13 ± 4.27, p=0.001, K; 11.77 ± 0.40 ng/mL vs H-LV400+L; 51.08 ± 0.99, p=0.002; Figure 4). These results indicate that the application of the β3-AR antagonist L at a concentration of 0.1 µM, did not exert the expected inhibitory effect. The gene expressions of *AQP7* and *PLIN1* in all cell groups are shown in Figure 3.

DISCUSSION

In vitro models, particularly preadipocyte cell lines such as 3T3-L1 cells, are widely used to study the processes of adipocyte proliferation, differentiation, adipokine secretion, and gene/protein expression. Under high-glucose conditions, the 3T3-L1 cell line undergoes hypertrophy and becomes insulin-resistant. These cells have been extensively utilised to gain a deeper understanding of the fundamental cellular processes relating to obesity and associated disorders (3, 25). *In vitro* experiments using the 3T3-L1 model have demonstrated that phytochemicals, including polyphenols, induce adipose tissue browning by reducing adipogenesis/lipogenesis or enhancing lipolysis/SYA oxidation, thereby producing an anti-obesity effect. This study was designed to assess the potential effects of LV polyphenol on the expression of the *AQP7* and *PLIN1* genes in response to molecules that simulate lipogenesis/lipolysis. Despite the initial expectations, both doses of LV decreased *AQP7* and *PLIN1* gene expression and reduced glycerol output. However, administration of a low dose of LV in combination with CL, which mimics β3-AR activation, led to an increase in *AQP7* and *PLIN1* gene expressions, as well as glycerol levels in the cell medium. These findings suggest that the expected lipolytic effects of LV are mediated through β3-AR activation.

Polyphenols, renowned for their pharmacological properties, are among the most extensively researched natural compounds. A typical diet contains over 500 different polyphenols. Although extracts simplify this variety into a complex mixture, the relationship between this complexity and human health remains unclear. Several recent studies have suggested that polyphenols have a significant effect on obesity, a condition that is becoming increasingly common. Adipocyte hypertrophy impairs cellular functions, elevates

reactive oxygen species (ROS) production, and contributes to inflammation, thereby worsening obesity-related metabolic disorders (26). Polyphenols found in LVs have been shown to inhibit several damaging effects commonly associated with obesity (9). LV polyphenols reduce lipid accumulation in adipocytes, resulting in cellular ROS production (8). These findings were validated in an animal model of diet-induced obesity (9). The LV polyphenol doses used in our study were determined based on the quantities described in the research carried out by M. Herranz-López et al. (9).

Accumulating evidence indicates that *AQP7*, a glycerol channel, significantly influences glycerol availability in tissues where it is expressed. A deficiency of *AQP7* in adipose tissue is linked to increased triglyceride (TG) accumulation and the onset of obesity (15). In addition, *AQP7* overexpression contributes to insulin resistance in hypertrophic adipocytes (19, 27, 28). In our study, the increased expression of *AQP7* in hypertrophied 3T3-L1 cells was consistent with the literature. While targeting *AQP7* in anti-obesity treatment is being considered, several studies, including those on other tissues in which *AQP7* is expressed, are needed.

In this study, the effects of LV polyphenol, which plays a role in the regulation of lipolysis in 3T3-L1 hypertrophic cells, on *AQP7, PLIN1,* and glycerol levels were evaluated. The regulatory mechanism in humans and rodents is unknown. In addition, the results would have benefited from the western blot method. Further studies are necessary to explain interspecies regulation.

PLIN1 serves as a protective barrier against lipid droplets in adipocytes, shielding them from the effects of natural lipases, including HSL. In addition, it inhibits the movement of *AQP7* to the plasma membrane in the presence of feeding. *PLIN1* gene expression is known to be high in obese human and animal models (29). As reported in the literature, *PLIN1* expression was increased in the hypertrophied model.

β3-AR is acknowledged as a validated therapeutic target for obesity and associated metabolic disorders (30, 31). CL, a selective systemic β3-AR agonist, has been demonstrated to stimulate UCP-1 expression in adipose tissue and exert anti-obesity effects (32, 33). This study is the first to investigate the effects of CL, a β3-AR agonist, and LV polyphenol, a β3-AR antagonist, on the gene expressions of *AQP7* and *PLIN1*, as well as glycerol levels and release, in hypertrophic adipocyte cells and high glucose-induced insulin-resistant hypertrophic adipocytes. CL, which mimics the β3-AR receptor agonist, yielded different dose-dependent results when administered with LV polyphenol. Coadministration of CL with a low dose of LV polyphenol was found to be more effective in the formation of lipolytic conditions. *AQP7* gene expression is positively regulated by fasting and PPARγ agonists, (21, 36), whereas factors such as nutrition, increased circulating insulin levels, dexamethasone administration, and tumour necrosis factor-α (TNF-α) suppress *AQP7* gene expression (35). Administration of L, which mimics the activity of β3-AR antagonists, reduced the expression of *the AQP7* and *PLIN1* genes in hypertrophic adipocyte cells. Furthermore, coadministration of LV polyphenol at a dose of 400 µg/mL suppressed *AQP7* gene expression. However, it led to an unexpected increase in the glycerol content of the cell medium, and the cause of this phenomenon remains unclear. This situation requires further investigation for better clarification. The regulation of *AQP7* gene expression and glycerol levels in adipose tissue could be a key determinant of fat storage and overall glucose regulation in the body (11, 14, 18).

Few studies have investigated polyphenol interactions with *AQP7* and *PLIN1*. Apple polyphenols, a distinct group of polyphenolic compounds, have been demonstrated to inhibit adipose tissue formation in Wistar rats (36). and to decrease triglyceride absorption by inhibiting pancreatic lipase activity in both mice and humans (37). Furthermore, a study on diet-induced obesity in Wistar rats demonstrated that supplementation with apple polyphenols prevented an increase in adipose tissue by inhibiting adipose tissue hypertrophy (20). Apple polyphenol consumption resulted in higher *AQP7* expression in epididymal adipose tissue, potentially clarifying the rise in glycerol release observed through *ex vivo* lipolysis testing as well as the decrease in adipose tissue volume observed in apple polyphenol-fed rats (20). Similarly, the low-dose LV used in this study and β3-AR activation together increased *AQP7* gene expression and glycerol content. These results indicate that the beneficial effects of LV therapy are triggered by the activation of β3-AR (with effects such as fasting, cold exposure, and exercise). The Dietary polyphenols are potential therapeutic agents for the management of obesity (38).

Additional studies are needed to pinpoint the specific metabolites responsible for the anti-obesity properties of LV extract, thereby aiding the development of polyphenolic blends for potential therapeutic applications in obesity management. Further analysis of the identified metabolites could provide insights into their potential health benefits in humans.

CONCLUSION

Although the mechanisms of action of polyphenols remain unclear, their beneficial effects are not only mediated by their antioxidant properties. *AQP7* modulation in adipose tissue is a potential innovative therapeutic approach. The identification of *AQP7* inhibitors or polyphenols, which are largely found in plant-derived foods, represents a healthy and innovative approach to the prevention and treatment of metabolic diseases, including obesity. Further studies are required to explain the mechanisms of action more clearly.

Ethics Committee Approval: Ethical approval is not applicable as cell line is used.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- O.P., E.K.T., A.C.; Data Acquisition O.P., E.K.T., D.S.; Data Analysis/ Interpretation: O.P., E.K.T., J.T.; Drafting Manuscript- O.P., J.T., Critical Revision of Manuscript- O.P., E.K.T., D.S., J.T., A.C.; Final Approval and Accountability- O.P., E.K.T., D.S., J.T., A.C.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was funded by Trakya University, Scientific Research Projects Unit (TUBAP 2021-11).

REFERENCES

1. Zukiewicz-Sobczak W, Wróblewska P, Zwolinski J, Chmielewska-Badora J, Adamczuk P, Krasowska E et al. Obesity and poverty paradox in developed countries. Ann Agric Environ Med 2014; 21(3): 590-4.
2. Tchernof A, Despres JP. Pathophysiology of human visceral obesity: an update. Physiol Rev 2013; 93(1): 359-404.
3. Herranz-Lopez M, Fernandez-Arroyo S, Perez-Sanchez A, Barrajon-Catalan E, Beltran-Debon R, Menendez JA, et al. Synergism of plant-derived polyphenols in adipogenesis: perspectives and implications. Phytomedicine 2012; 19(3-4): 253-61.
4. Fernandez-Arroyo S, Herranz-Lopez M, Beltran-Debon R, Borras-Linares I, Barrajon-Catalan E, Joven J et al. Bioavailability of a polyphenol-enriched extract of *Hibiscus sabdariffa* in rats and associated antioxidant status. Mol Nutr Food Res 2012; 56(10): 1590-5.
5. Herranz-Lopez M, Olivares-Vicente M, Boix-Castejon M, Caturla N, Roche E, Micol V. Differential effects of a combination of *Hibiscus sabdariffa* and *Lippia citriodora* polyphenols in overweight/obese subjects: A randomised controlled trial. Sci Rep 2019; 9(1): 2999.
6. Amiot MJ, Riva C, Vinet A. Effects of dietary polyphenols on metabolic syndrome in humans: a systematic review. Obes Rev 2016; 17(7): 573-86.
7. Sánchez-Marzo N, Lozano-Sánchez J, Cádiz-Gurrea MdlL, Herranz-López M, Micol V, Segura-Carretero A. Relationships between the chemical structure and antioxidant activity of phytocompounds isolated from lemon verbena. Antioxidants 2019; 8(8): 324.
8. Joven J, Rull A, Rodriguez-Gallego E, Camps J, Riera-Borrull M, Hernández-Aguilera A et al. Multifunctional targets of dietary polyphenols in diseases: A case study of the chemokine network and energy metabolism. Food Chem Toxicol 2013; 51: 267-79.
9. Herranz-López M, Barrajón-Catalán E, Segura-Carretero A, Menéndez JA, Joven J, Micol V. Lemon verbena (*Lippia citriodora*) polyphenols alleviate obesity-related disturbances in hypertrophic adipocytes through AMPK-dependent mechanisms. Phytomedicine 2015; 22(6): 605-14.
10. da Silva IV, Rodrigues JS, Rebelo I, Miranda JPG, Soveral G. Revisiting metabolic syndrome: the emerging role of aquaglyceroporin. Cell Mol Life Sci 2018; 75(11): 1973-88.
11. Frühbeck G, Catalán V, Gómez-Ambrosi J, Rodríguez A. Aquaporin-7 and glycerol permeability as novel obesity drug-target pathways. Trends Pharmacol Sci 2006; 27(7): 345-7.
12. K. Ishibashi, S. Hara, and S. Kondo. Aquaporin water channels in mammals. Clin Exp Nephrol 2009; 13: 107-17.
13. Verkman AS, Anderson MO, Papadopoulos MC. Aquaporins: important but elusive drug targets. Nat Rev Drug Discov 2014; 13(4): 259-77.
14. da Silva IV, Soveral G. Aquaporins in obesity. In: Yang B, editor. Aquaporins. Advances in Experimental Medicine and Biology, vol 1398. Springer, Singapore; 2023.p.289-302.
15. Iena FM, Lebeck J. Implications of aquaglyceroporin 7 in energy metabolism. Int J Mol Sci 2018; 19(1): 154.
16. Brasaemle DL. The perilipin family of structural lipid droplet proteins: stabilisation of lipid droplets and control of lipolysis. J Lipid Res 2007; 48(12): 2547-59.
17. MacDougald OA, Burant CF. Obesity and metabolic perturbations after the loss of aquaporin 7, an adipose glycerol transporter. PNAS 2005; 102(31): 10759-60.
18. Rodriguez A, Catalan V, Gomez-Ambrosi J, Fruhbeck G. Role of aquaporin-7 in the pathophysiological control of fat accumulation in mice. FEBS Lett 2006; 580(20): 4771-6.
19. Fiorentini D, Zambonin L, Vieceli Dalla Sega F, Hrelia S. Polyphenols as modulators of aquaporin family in health and disease. Oxid Med Cell Longev 2015: 2015: 196914.
20. Boqué N, de la Iglesia R, de la Garza AL, Milagro FI, Olivares M, Bañuelos Ó, et al. Prevention of diet induced obesity by apple polyphenols in W istar rats via regulation of adipocyte gene expression and DNA methylation patterns. Mol Nutr Food Res 2013; 57(8): 1473-8.
21. Funes L, Laporta O, Cerdán-Calero M, Micol V. Effects of verbascoside, a phenylpropanoid glycoside from lemon verbena, on phospholipid model membranes. Chem Phys Lipids 2010; 163(2): 190-9.
22. Green H, Kehinde O. Establishment of a preadipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. Cell 1975; 5(1): 19-27.
23. Han CY, Kargi AY, Omer M, Chan CK, Wabitsch M, O'Brien KD, et al. Differential effects of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: dissociation of adipocyte hypertrophy from inflammation. Diabetes 2010; 59(2): 386-96.
24. Tsukatani T, Suenaga H, Shiga M, Ikegami T, Ishiyama M, Ezoe T et al. Rapid susceptibility testing of slowly growing nontuberculous mycobacteria using colorimetric microbial viability assays based on reduced water-soluble tetrazolium WST-1. Eur J Clin Microbiol Infect Dis 2015; 34(10): 1965-73.
25. Ji E, Jung M, Park J, Kim S, Seo C, Park K et al. Inhibition of adipogenesis in 3T3-L1 cells and suppression of abdominal fat accumulation in high-fat diet fed C57BL/6J mice after downregulation of hyaluronic acid. Int J Obes 2014; 38(8): 1035-43.
26. Han CY, Subramanian S, Chan CK, Omer M, Chiba T, Wight TN, et al. Adipocyte-derived serum amyloid A3 and hyaluronan play roles in monocyte recruitment and adhesion. Diabetes 2007; 56(9): 2260-73.
27. Shen FX, Gu X, Pan W, Li WP, Li W, Ye J, et al. Over-expression of AQP7 contributes to improve insulin resistance in adipocytes. Exp Cell Res 2012; 318(18): 2377-84.
28. Kishida K, Kuriyama H, Funahashi T, Shimomura I, Kihara S, Ouchi N et al. Aquaporin adipose, a putative glycerol channel in adipocytes. J Biol Chem 2000; 275(27): 20896-902.
29. Ardevol A, Blade C, Salvado MJ, Arola L. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. Int J Obes Relat Metab Disord 2000; 24(3): 319-24.
30. Pan R, Zhu X, Maretich P, Chen Y. Combating obesity with thermogenic fat: Current challenges and advancements. Front Endocrinol (Lausanne) 2020; 11: 185.

31. Mund RA, Frishman WH. Brown adipose tissue thermogenesis: beta3-adrenoreceptors as potential targets for the treatment of obesity in humans. Cardiol Rev 2013; 21(6): 265-9.
32. Ghorbani M, Himms-Hagen, J. Appearance of brown adipocytes in white adipose tissue during CL 316,243-induced reversal of obesity and diabetes in Zucker fa/fa rats. Int J Obes Relat Metab Disord 1997; 21(6): 465-75.
33. Clookey SL, Welly RJ, Shay D, Woodford ML, Fritsche KL, Rector RS, et al. Beta-3 adrenergic receptor activation rescues metabolic dysfunction in female estrogen receptor alpha-null mice. Front Physiol 2019; 10:9.
34. Kishida K, Shimomura I, Nishizawa H, Maeda N, Kuriyama H, Kondo H et al. Enhancement of the aquaporin adipose gene expression by a peroxisome proliferator-activated receptor gamma. J Biol Chem 2001; 276(51): 48572-9.
35. Fasshauer M, Klein J, Lossner U, Klier M, Kralisch S, Paschke R. Suppression of aquaporin adipose gene expression by isoproterenol, TNFalpha, and dexamethasone. Horm Metab Res 2003; 35(4): 222-7.
36. Nakazato K, Song H, Waga T. Effects of dietary apple polyphenol on adipose tissues weights in Wistar rats. Experimental Animals 2006; 55(4): 383-9.
37. Sugiyama H, Akazome Y, Shoji T, Yamaguchi A, Yasue M, Kanda T, et al. Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. J Agric Food Chem 2007; 55(11): 4604-9.
38. Joven J, Micol V, Segura-Carretero A, Alonso-Villaverde C, Menendez JA. Bioactive food components p. polyphenols and the modulation of gene expression pathways: can we the danger of chronic disease? Crit Rev Food Sci Nutr 2014; 54(8): 985-1001.

Experimed 2024; 14(2): 110-115

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1438153

Association of XRCC3 Thr241Met Polymorphism with Renal Cell Carcinoma in a Turkish Population

Nur Cinar Sirin[1], Serhat Kilinc[2], Canan Kucukgergin[2], Parvana Mikailova[2], Selcuk Erdem[3],Oner Sanli[3], Faruk Ozcan[3], Sule Seckin[2]

[1]Department of Biochemistry, Institute of Health Sciences, Istanbul University, Istanbul, Turkiye
[2]Department of Biochemistry, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye
[3]Division of Urologic Oncology, Department of Urology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkiye

ORCID ID: N.C.S. 0000-0001-5216-0952; S.K. 0009-0002-0483-7599; C.K. 0000-0002-1797-5889; P.M. 0000-0002-6883-9051; S.E. 0000-0002-3514-1394; O.S. 0000-0001-5801-6898; F.O. 0000-0001-7544-6638; S.S. 0000-0002-5496-4169

Cite this article as: Cinar Sirin N, Kilinc S, Kucukgergin C, Mikailova P, Erdem S, Sanli O, Ozcan F, Seckin S. Association of XRCC3 Thr241Met polymorphism with renal cell carcinoma in a Turkish population. Experimed 2024; 14(2): 110-115.

ABSTRACT

Objective: Renal cell carcinoma (RCC) is considered as a major type of cancer of the kidney and is estimated to account for about 2-3% of all malignancies in adults. DNA repair mechanisms play a crucial role in defending genomic integrity against DNA damage, and defects in DNA repair mechanisms are associated with cancer susceptibility. The XRCC3 gene plays a pivotal role in the DNA repair system through homologous recombination and chromosomal activity. Therefore, our research aimed to clarify whether XRCC3 Thr241Met polymorphism affects the initiation and progression of RCC.

Materials and Methods: This study included 129 patients with RCC and 212 healthy individuals. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was employed to analyze XRCC3 Thr241Met gene polymorphism using SPSS 23 software to facilitate data analysis.

Results: Our results revealed no remarkable differences in the genotype and allele frequencies of the Thr241Met polymorphism of XRCC3 between the patient and control groups. However, a greater risk of RCC was reported for the variant (Met/Met) genotype of XRCC3 gene polymorphism, particularly among smokers.

Conclusion: Although the XRCC3 Thr241Met polymorphism may not significantly influence RCC initiation and progression in a Turkish population, smoking seems to amplify the risk associated with the (Met/Met) genotype of XRCC3 gene polymorphism.

Keywords: Renal cell carcinoma, XRCC3, polymorphism, Thr241Met, PCR-RFLP.

INTRODUCTION

Renal cell carcinoma (RCC), which originates from renal epithelial cells, is the 16th most prevalent cancer, globally. According to GLOBOCAN data, 431,519 new cases were detected in 2020, constituting 2.2% of all malignancies (1, 2).

The etiology of RCC is multifactorial, with age, sex, obesity, high blood pressure, exposure to harmful chemicals at work, smoking, alcohol use, and their interactions being significant contributors (3, 4). Genetic variations, specifically single-nucleotide polymorphisms (SNPs), may also influence RCC risk. These DNA sequence variations may alter gene functions involved in DNA repair. Certain SNPs may be correlated with RCC risk modulation, treatment responsiveness, and patient survival (5).

DNA damage repaired by DNA repair systems may stem from a variety of factors, including environmental factors, internal metabolic reactions, errors during DNA replication, and random mutations. Disruption of DNA integrity may modify gene function and potentially lead to pathological conditions, including cancer (6). Among the most harmful forms of DNA damage are double-stranded DNA breaks (DSBs), which involve the cleavage of both DNA strands. Cells utilize nonhomologous end-joining

Corresponding Author: Canan Küçükgergin **E-mail:** ckgergin@istanbul.edu.tr
Submitted: 17.05.2024 **Revision Requested:** 07.08.2024 **Last Revision Received:** 07.08.2024 **Accepted:** 12.08.2024

(NHEJ) and homologous recombination (HR) as the main repair mechanisms for DSBs (7). These repair mechanisms involve different proteins and enzymes that recognize, process, and bring together destroyed ends of DNA strands.

The X-ray Repair Cross Complementing/3 (XRCC3) protein, a component of the Rad51-related family—which consists of XRCC2, XRCC3, RAD51B, RAD51C, and RAD51D—comprises 346 amino acids and participates in HR repair system (8). The HR repair system that is critical for maintaining genomic stability is a process that utilizes homologous DNA fragments as a template to restore DSBs (9). XRCC3 collaborates with RAD51C to form a complex known as CX3, and this complex acts downstream of RAD51 recruitment. RAD51C also binds RAD51B to join with RAD51D-XRCC2 dimer to form BCDX2 complex (10).

It has been indicated that XRCC3 Thr241Met (rs861539) gene polymorphism in exon 7 of the XRCC3 DNA repair gene results in a single nucleotide alteration from cytosine (C) to thymine (T). The exchange of threonine with methionine at codon 241 may modify the role of XRCC3 and its cooperation with other proteins (11). This polymorphism may alter the protein structure and consequently the efficiency of DNA repair (12). The inefficiency of HR repair mechanisms that leads to the accumulation of mutations may contribute to carcinogenesis. Studies have shown that XRCC3 gene polymorphism is linked to a variety of cancers, such as leukemia (13), gastric (14), lung (15), and colorectal cancer (16, 17). However, previous studies showing the influence of XRCC3 Thr 241Met polymorphism on RCC are limited. Therefore, in our study, we aimed to establish the correlation between the XRCC3 DNA repair gene variants and the initiation and clinicopathological factors of RCC in a Turkish population.

MATERIALS AND METHODS

Study Participants

Our study included 129 individuals (65.1% male and 34.9% female) diagnosed with RCC according to histopathological findings. Patients underwent either radical or partial nephrectomy at the Istanbul University, Istanbul Faculty of Medicine, Department of Urology. Each participant provided comprehensive information through a detailed questionnaire.

The control group was composed of individuals without cancer and compared with the patient group according to sex, age, body mass index (BMI), and smoking habits. There was no cancer history in the families of both patient and control groups. Classification of smoking status was based on a threshold of 20 packs/year, with those exceeding 20 packs/year was considered as smokers. On the other hand, individuals who smoked less than one cigarette/day or less than one year along their lifespan were considered as non-smokers. The staging of tumors was indicated based on tumor node metastasis (TNM) of the American Joint Committee on Cancer (AJCC), while tumor grade was defined using the Fuhrman grading system. RCC patients were categorized into localized (I+II) and advanced (III+IV) disease groups according to TNM staging. Based on the Fuhrman system, patients were categorized into low (I+II) and high (III+IV) grades. Ethical consent for the study was granted by Ethics Committee of Istanbul University, Faculty of Medicine, with informed permission acquired from all patients with RCC.

Genotyping

In this study, blood specimens were obtained from all participants, both patients with RCC and healthy controls, using tubes with EDTA. DNA was then isolated from the collected whole blood samples using the DNA extraction kit (Roche Diagnostics, Mannheim, Germany) and preserved at -20 °C for subsequent polymerase chain reaction (PCR) analysis.

Genotyping of the XRCC3 Thr 241Met (rs861539) polymorphism was performed according to the PCR-restriction fragment length polymorphism (RFLP) method. The PCR mix included 25 µL reactions with 10 pmol of each primer, 0.2 mM of dNTPs, 2 mM of $MgCl_2$, 100 ng of genomic DNA, 10× PCR buffer at pH 8.8, and 1.25 U of Taq polymerase (MBI Fermentas). The PCR cycling condition for XRCC3 was initial denaturation of 95°C for 5 min. followed by 30 cycles of denaturation at 84°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min and finally 5 min at 72°C. The 456-bp product was digested with Hsp92 restriction enzyme (Thermo Fisher Scientific), incubated overnight at 37°C (18). Fragment patterns for XRCC3 Thr 241Met genotypes were Thr/Thr (456 bp), Thr/Met (456 bp, 315 bp, 141bp), and Met/Met (315 bp, 141 bp). The digested products were subjected to gel electrophoresis on a 2% agarose gel and were subsequently stained with ethidium bromide to enable visualization under UV illumination.

Statistical Analyses

The statistical analysis was conducted with SPSS 23 software (SPSS-Inc, Chicago/ IL, USA). To assess mean differences between controls and RCC patients, Mann-Withney U test was utilized. The genotype distributions and allele frequencies were compared using chi-square tests. Odds ratios (ORs) and 95% confidence intervals (CI) were determined using logistic regression analysis. Verification of the Hardy–Weinberg equilibrium (HWE) for genotype distributions was accomplished using the chi-square test. The power of the study was assessed using "NCSS-2000" software (NCSS-Inc, Kaysville, UT, USA), aiming to define a size effect of 0.20 with two degrees of freedom at an alpha level of 0.05 (α: 0.05), achieving a power of 90%.

RESULTS

Table 1 represents clinical and demographic parameters of the RCC patient and control groups. Any remarkable differences were not detected between controls and patients with RCC according to age, sex, BMI, and smoking status. Disease staging showed that 65.2% of the RCC-patients were diagnosed at low stage (I+II), whereas 34.8% were at high stage (III+IV).

Table 1. Clinical and demographic parameters of the controls and RCC patients.

Parameters	Controls (n=212)	Patients (n=129)	[a]p value
Age (years; mean ± SD)	56.0 ± 9.07	54.7 ± 10.5	0.250
BMI (kg/m^2; mean ± SD)	27.2 ± 2.56	27.7 ± 4.46	0.178
Sex (%; Female /Male)	68 (32.1)/ 144 (67.9)	45 (34.9)/ 84 (65.1)	0.593
Smoking status (%; Never/Current)	65.4/34.6	55.8/44.2	0.077
Grade (n, %)			
I		27 (20.9)	
II		57 (44.2)	
III		31 (24.0)	
IV		14 (10.9)	
Stage (n, %)			
I		78 (60.5)	
II		6 (4.7)	
III		38 (29.5)	
IV		7 (5.3)	
Histology (n, %)			
Clear cell		102 (79.1)	
Papillary		11 (8.5)	
Chromophobe		16 (12.4)	

[a]p from Pearson's χ^2 test for categorical variables and Mann–Whitney U or Student's t-test for continuous variables. n: Number of subjects.

Moreover, 65.1% of the patients were classified as having a low grade(I+II) of the disease, whereas 34.9% had a high stage (III+IV), indicating a predominance of low stage and low grade diagnoses among RCC patients.

Table 2 includes the genotype and allele frequencies of Thr241 Met polymorphism of the XRCC3 gene in both controls and patients with RCC. The comparative analysis revealed that no significant differences were observed in the genotype distributions and allele frequencies of Thr241Met polymorphism of XRCC3 between controls and patients with RCC.

On the other hand, we assessed allele frequencies and genotype distributions in relation to RCC susceptibility among smokers versus non-smokers. According to our findings, carriers of Met/ Met genotypes exhibited a 2.22-fold increased risk (OR=2.22; 95%CI=1.10-4.47; p=0.025) of having RCC compared with wild-type (Thr/Thr) carriers among smokers. However, the variant (Met/ Met) genotype distribution was not observed to be high among non-smokers (OR=0.99; 95%CI=0.60-1.63; p=0.970; Table 3).

In addition, we examined the impact of Thr241Met polymorphism of XRCC3 on clinicopathological features, such as grade and T stage among RCC patients. In our study, we did not find any statistically significant association between the clinicopathological characteristics of RCC and this polymorphism, including T stage and grade among RCC patients (Table 4).

Table 2. Genotype and allele frequencies of XRCC3 Thr241 Met gene polymorphism in controls and RCC patients.

	Controls n (%)	Patients n (%)	p value	OR[a] (95% CI)
XRCC3 Thr241Met				
Thr/Thr	48 (22.6)	27 (20.9)		1.00*
Thr/Met	117 (55.2)	59 (45.7)	0.981	0.99 (0.51-1.92)
Met/Met	47 (22.2)	43 (33.3)	0.777	1.01 (0.90-1.14)
Thr/Met+Met/ Met	164 (77.4)	102 (79)	0.604	0.98 (0.96-1.01)
Allele				
Thr	213 (50.2)	113 (43.7)		1.00*
Met	211 (49.7)	145 (56.2)	0.102	1.29 (0.94-1.76)

[a]Odds ratios (OR) and 95% confidence intervals (CI) adjusted for age, sex, BMI, and smoking status

*Reference genotype. n: Number of subjects.

DISCUSSION

RCC is more frequently observed in males than females, and it is the predominant form of cancer affecting the urinary system, with a mortality rate ranging from 30% to 40%. In addition, chronic kidney disease, hypertension, smoking, and obesity are risk factors for RCC occurrence (19).

Genetic defects in DNA repair mechanisms frequently lead to an elevation in various types of cancer, including RCC. HR is an essential repair process for maintaining genomic stability against DSBs (14, 20). The Rad51 complex is known to exhibit DNA-induced ATPase activity and binds to single-stranded DNA. The presence of XRCC3 protein is essential for the stimulation of Rad51 complex formation within cells (11). There is a growing evidence that XRCC3 maintains genomic stability and prevents the generation of tumorigenic mutations in the HR repair pathway. The Thr241Met polymorphism is located within the ATP-binding domain of the XRCC3 gene, which is crucial for its function in DNA repair. The transition from threonine to methionine at 241 position has been implicated in reduced efficacy in repairing chromosomal aberrations caused by X-rays, likely due to alterations in the structure of the

Table 3. Genotype and allele frequencies of XRCC3 Thr 241Met polymorphism associated with smoking status.

		Controls n (%)	Patients n (%)	p value	OR[a] (95% CI)
Non-smokers	**XRCC3 Thr(241)Met**				
	Thr/Thr	32 (23.2)	17 (23.6)		1.00*
	Thr/Met	71 (51.1)	30 (41.7)	0.850	0.91 (0.38-2.22)
	Met/Met	36 (25.9)	25 (34.5)	0.970	0.99 (0.60-1.63)
	Thr/Met+Met/Met	107 (77.0)	55 (76.2)	0.884	0.94 (0.41-2.13)
	Allele				
	Thr	135 (48.5)	64 (44.4)		1.00*
	Met	143 (51.4)	80 (55.5)	0.610	1.12 (0.71-1.77)
Smokers	**XRCC3 Thr(241)Met**				
	Thr/Thr	15 (20.5)	10 (17.5)		1.00*
	Thr/Met	47 (64.4)	29 (50.9)	0.711	1.22 (0.41-3.60)
	Met/Met	11 (15.1)	18 (31.6)	0.025	2.22 (1.10-4.47)
	Thr/Met+Met/Met	58 (79.5)	47 (82.5)	0.498	1.41(0.51-3.86)
	Allele				
	Thr	77 (54.7)	49 (42.9)		1.00*
	Met	69 (47.6)	65 (57.0)	0.118	1.48 (0.90-2.42)

[a]Odds ratios(OR) and 95% confidence intervals(CI) adjusted for age, sex, and BMI; *:Reference genotype. n: Number of subjects.

XRCC3 protein (21). Therefore, it has been hypothesized that the exchange generated by Thr241Met polymorphism in the XRCC3 gene is related to disruption of the connectivity with the Rad51 protein involved in DNA repair (22).

XRCC3 gene polymorphism has been the subject of global investigation for its possible links to various cancers. Previous research by Osawa et al. (15) highlighted the influence of XRCC3 gene polymorphism on long-term outcomes in patients with lung cancer. A meta-analysis by Qin et al. (14) demonstrated a significant elevation in gastric cancer susceptibility among Asian populations due to XRCC3 gene polymorphism. According to Xie et al. (13), the Thr241Met polymorphism was found related to the incidence of leukemia in a Caucasian population. Wang and Zhang (17) and Procopciuc and Osian (16) reported that XRCC3 gene polymorphism may elevate the occurrence of colorectal cancer. Based on these findings, we presumed that XRCC3 gene polymorphism may also be involved in the risk of RCC in a Turkish population. According to our current knowledge, there exists no documented report investigating Thr241Met polymorphism of XRCC3 and its relationship with RCC susceptibility in a Turkish population. However, based on our study findings, (Met/Met) and (Thr/Met) genotypes of XRCC3 did not alter the risk of RCC. Our study outcomes were similar to the results obtained by Loghin et al. (23), who reported no relation of increased clear cell-renal cell carcinoma with Met/Met genotype of XRCC3 gene polymorphism in a Romanian population. In addition, Hirata et al. (24) did not detect any correlation between heterozygous (Thr/Met) genotype of XRCC3 gene polymorphism and RCC risk.

It has been demonstrated that tobacco consumption, including several harmful chemicals that modify DNA structure, may lead to mutations (25). Therefore, tobacco usage may be a major risk factor for various cancers, such as RCC. Many prior studies have demonstrated a significant relationship between XRCC3 gene polymorphism and smoking, such as colorectal (16, 26), bladder (27), lung (14), and breast cancer (28). Similar to previous studies, our results showed that the variant genotype of the XRCC3 gene was significantly linked to an elevated risk of RCC among smokers.

When stratified by disease stage and grade, the risk genotype (Met/Met) did not affect tumor staging and grading among patients with RCC. Our findings are in accordance with those of Loghin et al. (23) who determined no relationship between genotype distributions and clinicopathological factors of CCRCC.

Table 4. Genotype and allele frequencies of the Thr 241Met polymorphism of XRCC3 according to disease grade and stage.

	Low grade[a] n (%)	High grade[b] n (%)	p value	OR[e] (95% CI)
XRCC3 Thr 241 Met				
Thr/Thr	14 (16.7)	13 (28.9)		1.00*
Thr/Met	44 (52.4)	15 (33.3)	0.052	0.36 (0.14-0.95)
Met/Met	26 (31.1)	17 (37.8)	0.479	0.83 (0.51-1.36)
Thr/ Met+Met/ Met	70 (83.5)	32 (71.1)	0.103	0.49 (0.20-1.16)
Allele				
Thr	72 (42.8)	41 (45.5)		1.00*
Met	96 (57.1)	49 (54.4)	0.677	0.99 (0.53-1.30)
	Low stage[c] n (%)	**High stage[d] n (%)**	**p value**	**OR[e] (95% CI)**
XRCC3 Thr 241 Met				
Thr/Thr	17 (20.2)	10 (22.2)		1.00*
Thr/Met	41(48.8)	18 (40.0)	0.418	0.06 (0.21-1.90)
Met/Met	26 (31.0)	17 (37.8)	0.353	0.70 (0.34-1.46)
Thr/ Met+Met/ Met	67 (79.8)	35 (77.8)	0.392	0.63 (0.21-1.81)
Allele				
Thr	75 (44.5)	38 (42.2)		1.00*
Met	93 (55.3)	52 (57.7)	0.708	1.10 (0.65-1.85)

[a]Low grade(I-II); [b]High grade (III-IV); [c]Low stage (I-II); [d]High stage (III-IV) [e]Odds ratios (OR) and 95% confidence intervals(CI) adjusted for age, sex, BMI, and smoking status.
*Reference genotype. n: Number of subjects.

Although the current study has some novel findings, not evaluating the relationship between genotype distributions of other SNPs of XRCC3 gene and RCC was a limitation of our study. More SNPs of XRCC3 gene need to be studied in the future.

In conclusion, this study suggests no association between increased risk of RCC and genetic variants of the XRCC3 gene. However, smoking habits may influence the interaction between the variant genotype of XRCC3 gene polymorphism and the risk of RCC in a Turkish population. It is crucial to acknowledge that genetic polymorphisms may have different effects across different ethnic groups, and their contributions to cancer etiology are typically complex and multifactorial.

Acknowledgement: This work was supported by the Research Fund of Istanbul University. Project number: 55485.

Ethics Committee Approval: The study design was approved by the Ethics Committee of Istanbul University, Faculty of Medicine. (25.05.2015/1034).

Informed Consent: Informed consent was obtained from all participants included in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- N.C.S., S.K., C.K., P.M.; Data Acquisition S.E., O.S., F.O.; Data Analysis/ Interpretation: C.K.; Drafting Manuscript- N.C.S., S.K., C.K.; Critical Revision of Manuscript- S.S., C.K., Final Approval and Accountability- S.S., C.K., N.C.S.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: The authors declare that this study has received no financial support.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71(3): 209-49.
2. Hsieh JJ, Purdue MP, Signoretti S, Swanton C, Albiges L, Schmidinger M et al. Renal cell carcinoma. Nat Rev Dis Primers 2017; 3(1): 1-19.
3. Chow WH, Dong LM, Devesa SS. Epidemiology and risk factors for kidney cancer. Nat Rev Urol 2010; 7(5): 245-57.
4. Nelson EC, Evans CP, Lara Jr PN. Renal cell carcinoma: current status and emerging therapies. Cancer Treat Rev 2007; 33(3): 299-313.
5. Horikawa Y, Wood CG, Yang H, Zhao H, Ye Y, Gu J et al. Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma. Clin Cancer Res 2008; 14(23): 7956-62.
6. Sharma R, Lewis S, Wlodarski MW. DNA repair syndromes and cancer: insights into genetics and phenotype patterns. Front Pediatr 2020; 8: 570084.
7. Michalska MM, Samulak D, Romanowicz H, Jabłoński F, Smolarz B. Association between single nucleotide polymorphisms (SNPs) of XRCC2 and XRCC3 homologous recombination repair genes and ovarian cancer in Polish women. Exp Mol Pathol 2016; 100(2): 243-7.
8. Ozoran E, Trabulus FDC, Erhan D, Batar B, Guven M. Association of XRCC3, XRCC4, BAX, and BCL-2 polymorphisms with the risk of breast cancer. Int J Breast Cancer 2022; 2022: 5817841.

9. Liu Q, Peng Q, Zhang B, Tan Y. X-ray cross-complementing family: the bridge linking DNA damage repair and cancer. J Transl Med 2023; 21(1): 602.
10. Longo MA, Roy S, Chen Y, Tomaszowski KH, Arvai AS, Pepper JT et al. RAD51C-XRCC3 structure and cancer patient mutations define DNA replication roles. Nat Commun 2023; 14(1): 4445.
11. Bonilla B, Hengel SR, Grundy MK, Bernstein KA. RAD51 gene family structure and function. Annu Rev Genet 2020; 54: 25-46.
12. Matullo G, Palli D, Peluso M, Guarrera S, Carturan S, Celentano et al. XRCC1, XRCC3, XPD gene polymorphisms, smoking and 32 P-DNA adducts in a sample of healthy subjects. Carcinogenesis 2001; 22(9): 1437-45.
13. Xie Z, Peng W, Li Q, Cheng W, Zhao X. Ethnicity-stratified analysis of the association between XRCC3 Thr241Met polymorphism and leukemia: an updated meta-analysis. BMC Med Genomics 2021; 14(1): 1-11.
14. Qin XP, Zhou Y, Chen Y, Li NN, Wu XT. XRCC3 Thr241Met polymorphism and gastric cancer susceptibility: A meta-analysis. Clin Res Hepatol Gastroenterol 2014; 38(2): 226-34.
15. Osawa K, Nakarai C, Uchino K, Yoshimura M, Tsubota N, Takahashi J et al. XRCC3 gene polymorphism is associated with survival in Japanese lung cancer patients. Int J Mol Sci 2012; 13(12): 16658-67.
16. Procopciuc L, Osian G. Interaction between lifestyle factors and the XRCC1, XPD, and XRCC3 genetic variations modulates the risk for sporadic colorectal cancer. Rev Rom Med Lab 2014; 22(1): 129-41.
17. Wang Z, Zhang W. Association between XRCC3 Thr241Met polymorphism and colorectal cancer risk. Tumor Biology 2013; 34: 1421-29.
18. Le Marchand L, Donlon T, Lum-Jones A, Seifried A. Association of the hOGG1 Ser326Cys polymorphism with lung cancer risk. Cancer Epidemiol Biomarkers Prev 2022; 11: 409-12.
19. Bahadoram S, Davoodi M, Hassanzadeh S, Bahadoram M, Barahman M, Mafakher L. Renal cell carcinoma: An overview of the epidemiology, diagnosis, and treatment. G Ital Nefrol 2022; 39(3): 2022-vol3.
20. Alhmoud JF, Woolley JF, Al Moustafa AE, Malki MI. DNA damage/repair management in cancers. Cancers 2020; 12 (4): 1050.
21. Fan J, Liu W, Zhang M, Xing C. A literature review and systematic meta-analysis on XRCC3 Thr241Met polymorphism associating with susceptibility of oral cancer. Oncol Lett 2019; 18(3): 3265-73.
22. dos Santos Pereira J, Fontes FL, de Medeiros SRB, de Almeida Freitas R, de Souza LB, da Costa Miguel MC. Association of the XPD and XRCC3 gene polymorphisms with oral squamous cell carcinoma in a Northeastern Brazilian population: A pilot study. Arch Oral Biol 2016; 64, 19-23.
23. Loghin A, Bănescu C, Nechifor-Boila A, Chibelean C, Orsolya M, Nechifor-Boila A et al. XRCC3 Thr241Met and XPD Lys751Gln gene polymorphisms and risk of clear cell renal cell carcinoma. Cancer Biomarkers 2016; 16(2): 211-7.
24. Hirata H, Hinoda Y, Matsuyama H, Tanaka Y, Okayama N, Suehiro Y et al. Polymorphisms of DNA repair genes are associated with renal cell carcinoma. Biochem Biophys Res Commun 2006; 342(4): 1058-62.
25. Neumann AS, Sturgis EM, Wei Q. Nucleotide excision repair as a marker for susceptibility to tobacco-related cancers: a review of molecular epidemiological studies. Mol Carcinog 2005; 42(2): 65-92.
26. Improta G, Sgambato A, Bianchino G, Zupa A, Grieco V, La Torre G et al. A. Polymorphisms of the DNA repair genes XRCC1 and XRCC3 and risk of lung and colorectal cancer: a case-control study in a Southern Italian population. Anticancer Res 2008; 28(5B): 2941-6.
27. Zhu X, Zhong Z, Zhang X, Zhao X, Xu R, Ren W. et al. DNA repair gene XRCC3 T241M polymorphism and bladder cancer risk in a Chinese population. Genet Test Mol Biomark 2012; 16(6): 640-3.
28. Hu J, Wang N, Wang YJ. XRCC3 and RAD51 expression are associated with clinical factors in breast cancer. PLoS One 2013; 8(8): e72104.

Experimed 2024; 14(2): 116-125

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1489790

Capsaicin Modulates Adipocyte Cell Differentiation and Inflammatory Gene Expression

Sevgin Degirmencioglu[1], Pinar Cetinalp[2], Muhammed Seyithanoglu[3], Sevda Tanrikulu-Kucuk[2], Hikmet Kocak[4], Yildiz Oner-Iyidogan[5]

[1]Department of Medical Biochemistry, Faculty of Medicine, Kirklareli University, Kirklareli, Turkiye
[2]Department of Medical Biochemistry, Faculty of Medicine, Demiroglu Bilim University, Istanbul, Turkiye
[3]Department of Medical Biochemistry, Faculty of Medicine, Kahramanmaras Sutcu Imam University, Kahramanmaras, Turkiye
[4]Department of Medical Education, Faculty of Medicine, Istinye University, Istanbul, Turkiye
[5]Department of Medical Biochemistry, Faculty of Medicine, Istanbul Arel University, Istanbul, Turkiye

ORCID ID: S.D. 0000-0001-7243-3671; P.C. 0000-0001-8105-0322; M.S. 0000-0002-8027-7549; S.T.K. 0000-0002-1173-7759; H.K. 0000-0003-1720-9102; Y.O.I. 0000-0001-6956-8794

Cite this article as: Degirmencioglu S, Cetinalp P, Seyithanoglu M, Tanrikulu-Kucuk S, Kocak H, Oner-Iyidogan Y. Capsaicin modulates adipocyte cell differentiation and inflammatory gene expression. Experimed 2024; 14(2): 116-125.

ABSTRACT

Objective: Adipose tissue stores lipids necessary for the maintenance of nutritional homeostasis. It is also an endocrine organ that reacts to changes in inflammation and energy status. Capsaicin, the principal bioactive compound in red pepper, has garnered significant attention for its reported anti-obesity, anti-diabetic, anti-oxidant, and anti-inflammatory properties. In this study, we aimed to elucidate the influence and most efficacious dose of capsaicin on the expression of lipid metabolism-related inflammatory proteins and the inhibition of adipocyte cell differentiation.

Materials and Methods: Cell viability analysis was performed using CCK-8, cell differentiation was assessed using Oil Red O, and gene expression levels of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (C/EBPα), adiponectin, leptin, cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), nuclear factor kappa B1 (NF-κB1), tumor necrosis factor-alpha (TNF-α), sirtuin-1 (SIRT-1), transient receptor potential vanilloid receptor 1 (TRPV1), and uncoupling protein 2 (UCP2) were evaluated using quantitative real time polymerase chain reaction (qRT-PCR). Statistical analyses were conducted using GraphPad Prism 5. One-way ANOVA was performed to compare quantitative data between the groups.

Results: Capsaicin suppressed preadipocyte-to-adipocyte differentiation and mitigated the release of pro-inflammatory cytokines, particularly at low concentrations. Capsaicin effectively suppressed adiponectin levels at all concentrations but decreased leptin levels at lower concentrations (0.5 μM and 1 μM). Capsaicin stimulated the expressions of SIRT1 and TRPV-1 in adipocytes. According to our findings, the most effective capsaicin dose for the regulation of SIRT1 and TRPV-1 expressions appears to be 20 μM.

Conclusion: Capsaicin's effect on proteins regulating adipogenesis is not dose-related, but its inhibitory effect on adiposity-dependent inflammation was more pronounced at low concentrations.

Keywords: Adipogenesis, capsaicin, cytokines, differentiation and inflammation

INTRODUCTION

One of the common health problems worldwide is the increasing prevalence of obesity and obesity-related chronic conditions. The excessive storage of fat in adipose tissue and increase in body weight are the main causes of obesity. Adipose tissue plays a crucial role in controlling body weight, as it stores the lipids necessary for maintenance of nutritional homeostasis. Additionally, it is an endocrine organ that reacts to changes in inflammation and energy status (1).

Corresponding Author: Sevgin Degirmencioglu **E-mail:** sevgindegirmencioglu@klu.edu.tr
Submitted: 25.05.2024 **Revision Requested:** 02.07.2024 **Last Revision Received:** 31.07.2024 **Accepted:** 02.08.2024

Red pepper (Chili pepper, Capsicum annuum L.) is a flavoring spice used in food. It has been demonstrated that some of the active components of red pepper have many physiological roles (2). Capsaicin, the primary and extensively studied bioactive component of red pepper, has been documented to possess anti-obesity, anti-diabetic, anti-oxidant, and anti-inflammatory properties (2, 3). Many epidemiological and animal studies have shown that the anti-obesity effect of capsaicin is related to its potential to stimulate the transient receptor potential vanilloid receptor 1 (TRPV1) receptor. The mechanism by which capsaicin affects TRPV1 is not fully understood, but recent studies suggest that TRPV1 plays a significant role in the regulation of body weight, glucose, and lipid metabolism (4, 5). Zheng et al. have suggested that one of the potential mechanisms exposing the anti-obesity effects of capsaicin is to increase lipid oxidation and to inhibit adipogenesis (6). Studies have reported that capsaicin induces the transformation of white adipose tissue (WAT) into brown adipose tissue (BAT) through sirtuin-1 (SIRT-1) dependent deacylation (7). A recent study showed that capsaicin also has a receptor-independent effect, which can trigger nuclear factor κB (NF-κB) inactivation and prevent inflammation associated with adipose tissue (8).

Adipogenesis is an essential metabolic pathway for the storage of lipids in adipose tissue. Previous studies have suggested that the reduction of preadipocyte differentiation, proliferation, and lipogenesis could prevent obesity (9). Hsu et al. reported that capsaicin suppresses the expression of adipogenesis-related transcription factors such as peroxisome proliferator-activated receptor gamma (PPAR-γ) and CCAAT/enhancer binding protein alpha (C/EBP-α). It was also shown that capsaicin controls the protein expression of leptin and adiponectin, which are two hormones primarily produced by adipose tissue (6, 9). Uncoupling protein 2 (UCP2), a protein situated in the inner mitochondrial membrane responsible for regulating lipid metabolism, was found to be diminished both in the omental and subcutaneous adipose tissues among obese individuals (10-12).

Adipose tissue and lipid storage represent an insidious source of chronic low-grade inflammation (13, 14). Adipose tissue-related inflammation facilitates adipogenesis (15). Capsaicin is

Table 1: List of primers used for qRT–PCR

Gene Name	Gene bank #	Primer Sequences	bp
PPARG	NM_138712.5	F: 5′-AGGATGCAAGGGTTTCTTCCG-3′ R: 5′-CCGCCAACAGCTTCTCCTTC-3′	200
CEBPA	NM_001285829.1	F: 5′-CACCGCTCCAATGCCTACTG-3′ R: 5′-CTAAGGACAGGCGTGGAGGA-3′	200
NFKB1	NM_003998.4	F: 5′-ACTGCTGGACCCAAGGACAT-3′ R: 5′-CGCCTCTGTCATTCGTGCTT-3′	105
TNFA	NM_000594.4	F: 5′- AGAACTCACTGGGGCCTACA-3′ R: 5′- GCTCCGTGTCTCAAGGAAGT-3′	177
UCP2	NM_001381944.1	F: 5′-CTTCTGCACCACTGTCATCG-3′ R: 5′-GTGACGAACATCACCACGTT-3′	195
TRPV1	NM_080704.4	F: 5′-ACCCTGTTTGTGGACAGCTA-3′ R: 5′-CAAGGCCAGGGAGAATACCA-3′	129
SIRT1	NM_012238.5	F: 5′-TATGCTCGCCTTGCTGTAGA-3′ R: 5′-TGGCTGGAATTGTCCAGGAT-3′	132
COX2	NM_000963.4	F: 5′-GCTTCCATTGACCAGAGCAG-3′ R: 5′-CTCCACAGCATCGATGTCAC-3′	159
LEP	NM_000230.3	F: 5′-TGGAGAAGCTGATGCTTTGC-3′ R: 5′-GGACCATTCAGAGGGTCACA-3′	196
ADIPOQ	NM_001177800.2	F: 5′-GGATGTGAAGGTCAGCCTCT-3′ R: 5′-TACACCTGGAGCCAGACTTG-3′	141
GAPDH	NM_002046.7	F: 5′-ACCCAGAAGACTGTGGATGG-3′ R: 5′-TCAGCTCAGGGATGACCTTG-3′	124

PPARG: Peroxisome proliferator-activated receptor gamma, CEBPA: CCAAT/enhancer-binding protein, NFKB1: Nuclear factor-kappa B1, TNFA: Tumor necrosis factor alpha, UCP2: Uncoupling protein 2, TRPV1: Transient receptor potential vanilloid receptor-1, SIRT1: Sirtuin 1, COX2: Cyclooxygenase-2, LEP: Leptin, ADIPOQ: Adiponectin, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

an effective anti-inflammatory agent that helps to suppress the inflammatory process by reducing the expression of some pro-inflammatory proteins, such as tumor necrosis factor-alpha (TNF-α) and cytokines (16). However, the capacity of capsaicin to inhibit adipocyte differentiation and cellular inflammatory status is not yet well understood.

The primary aim of this study was to explore the influence of capsaicin on the expression of inflammatory markers associated with lipid metabolism, and to identify the most effective dosage.

MATERIALS AND METHODS

Cell Culture

Human preadipocyte cells (HPAd) from heart tissue were acquired from Cell Applications (San Diego, USA) and cultured in preadipocyte medium (Cell Applications, San Diego, USA) in 25 cm^2 tissue culture flasks from Corning (Charlotte, USA). The flask was then incubated for 24 h and placed in an incubator containing 5% CO_2 at 37°C (Sanyo, Osaka, Japan). In the following day, microscopy examination revealed that the cells had adhered to the flask and continued to proliferate in the subsequent days. When the cells reached 70-80% confluence, they were passaged according to the manufacturer's protocol (Cell Applications, San Diego, USA). The remaining procedures followed the methodology outlined in the experiment by Cetinalp et al. (17). On the 15th day, microscopy examination (Leica, Wetzlar, Germany) revealed that the cells contained granulated oil droplets and had successfully differentiated into adipocyte cells. 0.5μM, 1μM, 10μM, 20μM, and 50μM doses of capsaicin as ≥95% purity (Sigma, Taufkirchen, Germany) was applied.

Oil Red O Staining of Differentiated Adipocyte Cells to Quantify Lipid Accumulation

The cells were differentiated using Oil Red O (Sigma, Taufkirchen Germany) staining method. The remaining procedures followed the methodology outlined in the experiment by Cetinalp et al. (17).

Cell Viability Analysis Using CCK-8

Cell viability analysis was performed using a CCK-8 Kit (Abbkine, Georgia, USA). All groups were seeded with 15x10^3 cells per well in 6-well plates, transferred to a 96-well plate, and incubated at 37°C with 5% CO_2 for 1 day to allow the cells to attach to the plate. The following day, 10 μL of CCK-8 reagent was pipetted into all wells, and the plate was incubated at 37°C in an incubator containing 5% CO_2 for 3 h. After the incubation period, the absorbance of the wells was measured at 450 nm using a microplate reader (BioTek Instruments, Vermont, USA). Experiments were repeated three times for each group, and measurements were performed in triplicates.

Quantifying mRNA Expression in Cells Using qRT–PCR

For mRNA expression quantification, total RNA was extracted from the cells using RNAzol RT solution (MRC, Cincinnati, USA) according to the manufacturer's protocol. The concentrations and purities of the RNA samples were evaluated using a NanoDrop 2000 (Thermo Scientific, Massachusetts, USA). Prior to reverse transcription, RNA concentrations were standardized. Reverse transcription was performed using a Script cDNA Synthesis Kit (Jena Bioscience, Jena, Germany) following the kit protocol. The resulting cDNA was then amplified by quantitative

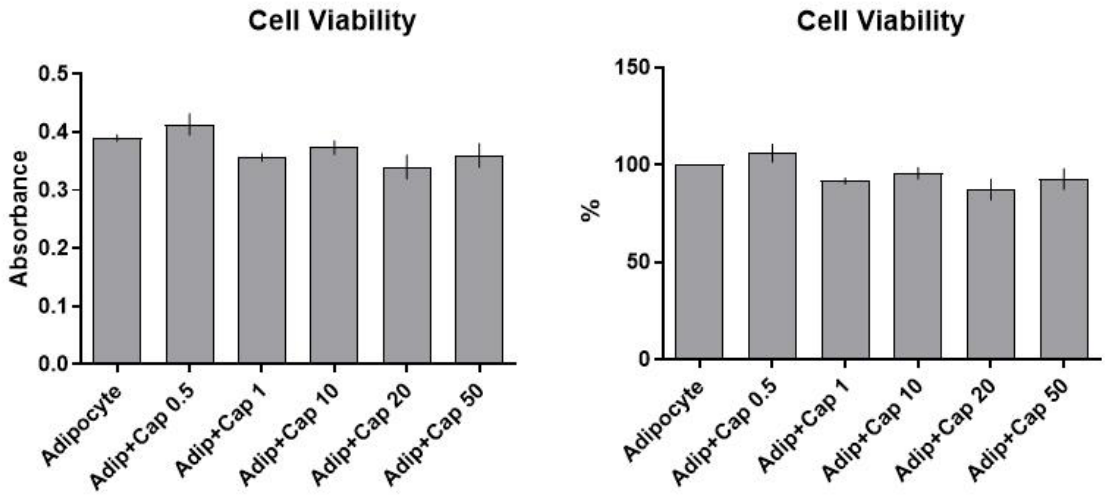

Figure 1. Cell viability assay following stimulation with different doses of capsaicin. Cap: Capsaicin ; Adip: Adipocyte.

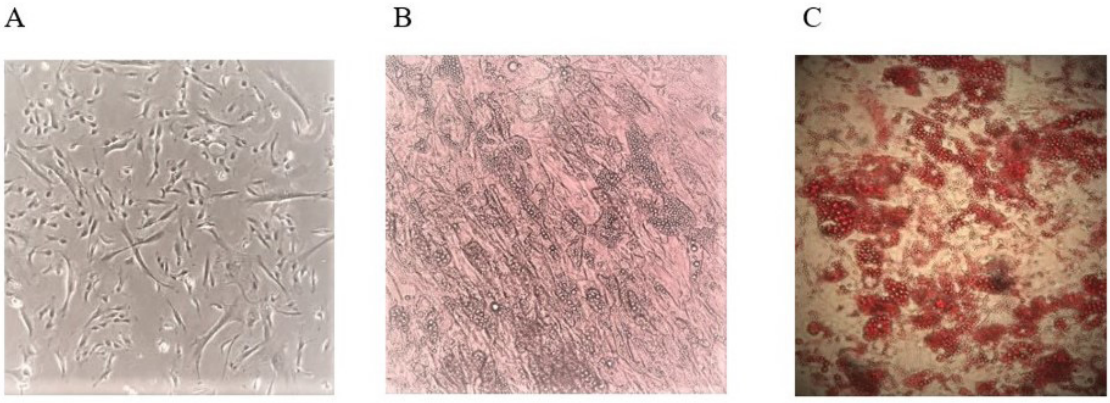

Figure 2. Differentiation of preadipocyte cells into mature adipocytes starting from day 0 and on day 15 and appearance of lipid droplets stained with Oil Red O dye under light microscopy. A. Preadipocyte, B. Mature adipocyte cells and C. Oil Red O staining of mature adipocyte cells.

reverse transcription polymerase chain reaction (qRT–PCR) using the qPCR GreenMaster with the UNG Kit (Jena Bioscience, Jena, Germany). The remaining procedures followed the methodology outlined in the experiment by Cetinalp et al. (17). The relative mRNA transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method and the relative expression of each gene was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Primers were sourced from LGC Standards (Middlesex, UK), and all qRT–PCR analyses were performed in triplicates. The primers used are detailed in Table 1.

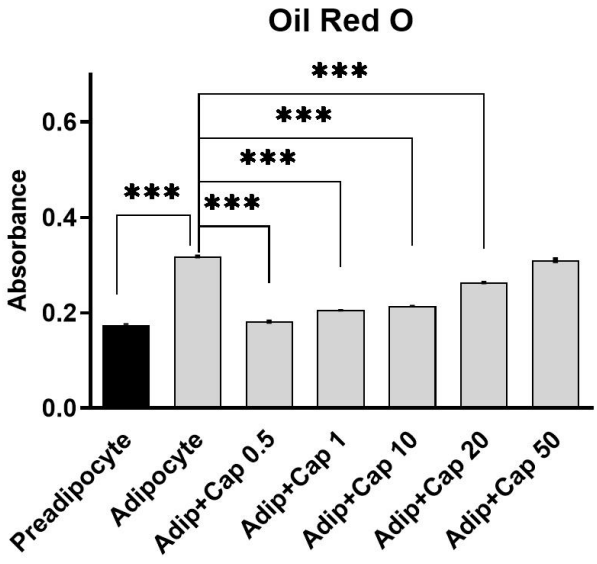

Figure 3. Comparison of the differentiation absorbance values of preadipocyte, adipocytes, and different doses of capsaicin-added adipocytes stained with Oil Red O dye. ***p<0.001; Cap: Capsaicin; Adip: Adipocyte.

Statistical Analyses

The results were presented as means ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 5 software (San Diego, USA). One-way ANOVA was performed to compare quantitative data between the groups. If the ANOVA results were significant, the Kruskal-Wallis Test was used to compare means between groups. The statistical significance level for all analyses was set at a significance level of $p<0.05$.

RESULTS

In this study, we investigated the molecular mechanisms responsible for the inhibitory effects of various capsaicin doses on differentiation. We used the CCK-8 assay to determine the cytotoxic effect of capsaicin at different doses and found no significant difference in cell viability ($p=0.0620$, Figure 1).

During this period, the morphology of the cells was examined under a microscope, and differentiation was determined on day 15. At the end of 15th day, Oil Red O staining method was applied for lipid accumulation in preadipocytes, adipocytes, and capsaicin-treated adipocytes (Figure 2). During differentiation, there was a notable increase in lipid accumulation in untreated adipocytes ($p<0.001$). Nevertheless, all capsaicin doses except for 50 µM inhibited lipid accumulation, presenting statistically significant differences compared with untreated adipocytes ($p<0.001$, Figure 3).

The effect of capsaicin on the expression of C/EBP-α and PPAR-γ genes

C/EBP-α levels experienced a notable increase in adipocytes during the differentiation process ($p<0.001$). However, treatment of cells with different doses of capsaicin (ranging from 0.5 to 50 µM) during differentiation resulted in reduced C/EBP-α levels in comparison with adipocytes ($p<0.001$, Figure 4).

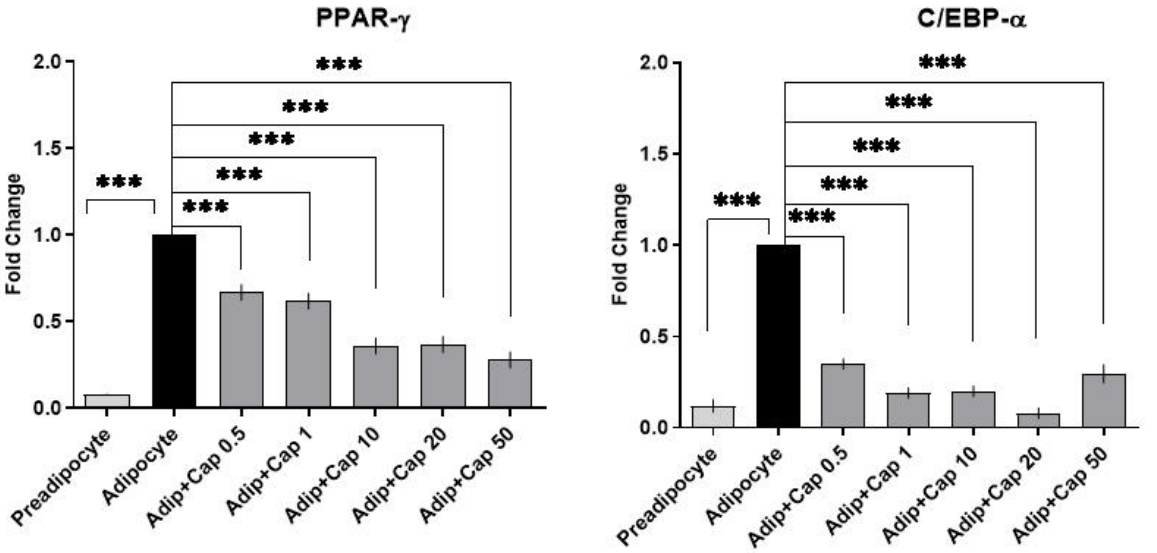

Figure 4. Effects of capsaicin on the expression of PPAR-γ (A) and C/EBP-α (B) during differentiation of preadipocyte to adipocytes ***p<0.001; Cap: Capsaicin; Adip: Adipocyte.

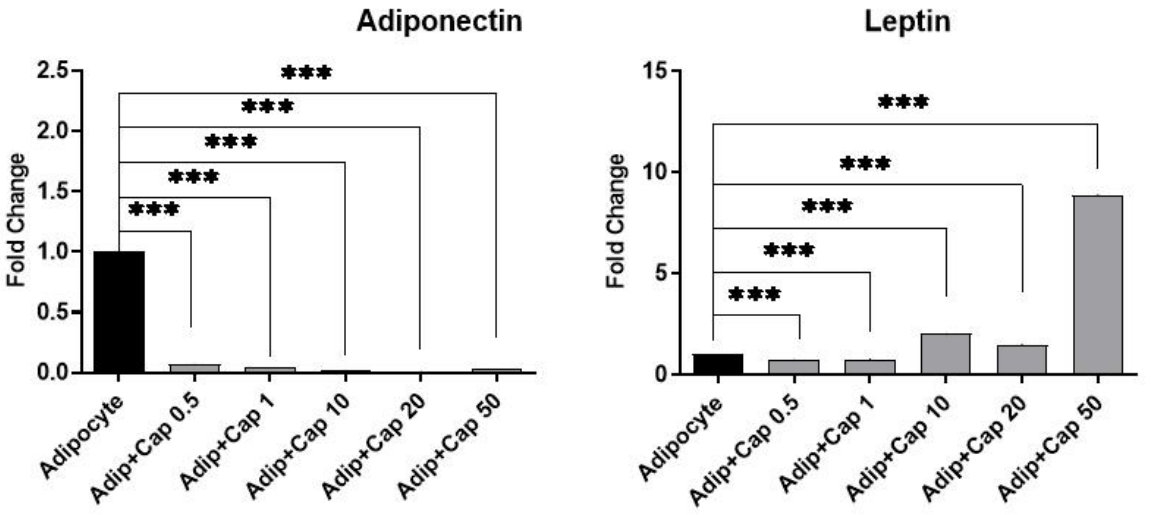

Figure 5. Effects of different doses of Capsaicin on adipokine gene expression. ***p<0.001, Cap: Capsaicin; Adip: Adipocyte.

Similarly, there was a significant increase in PPAR-γ expression in adipocytes during differentiation compared to preadipocyte. However, treatment with different concentrations of capsaicin (0.5-50 μM) during differentiation resulted in decreased PPAR-γ levels compared with adipocytes (p<0.001, Figure 4).

Impact of Capsaicin on Adipokine Gene Expression

Adiponectin levels exhibited a decrease in all capsaicin groups compared with adipocytes (p<0.001). However, no dose-dependent pattern was observed for the reduction of adiponectin levels (Figure 5).

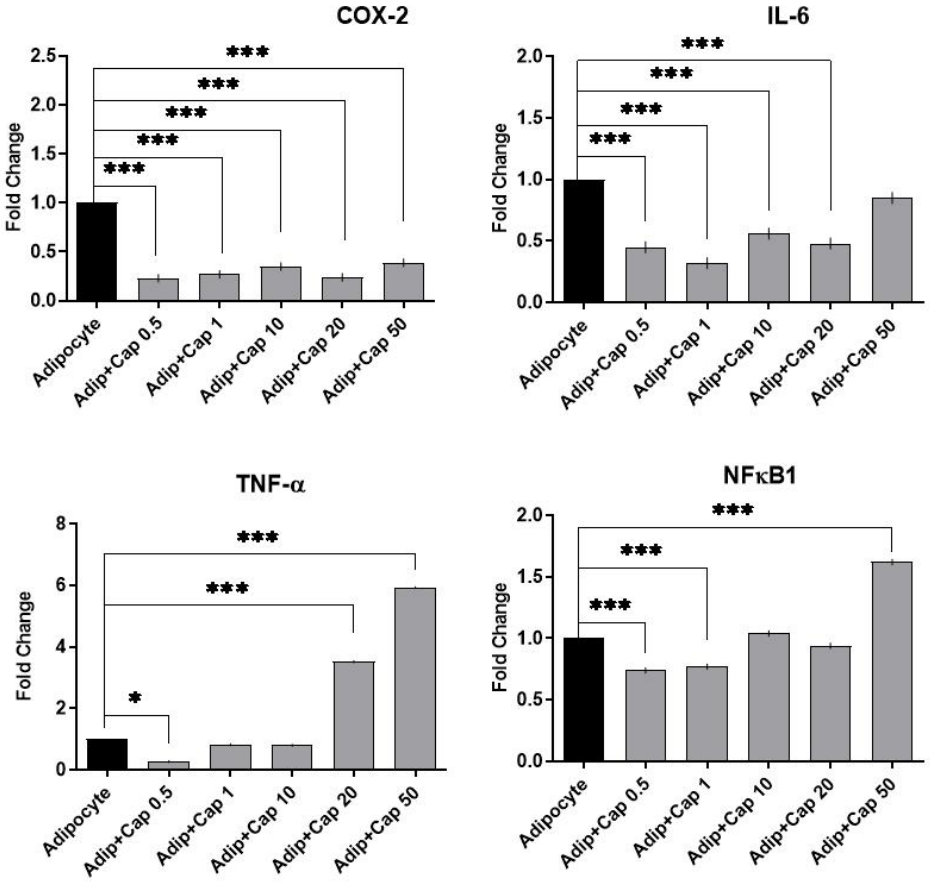

Figure 6. Effects of different doses of Capsaicin treatment to proinflammatory cytokines levels.
*p<0.05; ***p<0.001; Cap: Capsaisin; Adip: Adipocyte.

Leptin levels were significantly decreased in lower doses of capsaicin (0.5μM and 1μM) capsaicin-treated adipocytes and significantly increased in higher doses of capsaicin (10μM, 20μM, and 50μM) capsaicin-treated adipocytes in comparison with adipocytes (p<0.001, Figure 5).

Impact of Capsaicin on Proinflammatory Cytokines

In comparison with control adipocytes, COX-2 levels were significantly reduced in all adipocytes treated with capsaicin (p<0.001). Additionally, IL-6 gene expression levels were significantly decreased in all capsaicin-treated adipocytes except for the 50μM group when compared to untreated adipocytes (p<0.001). TNF-α levels were significantly increased in the capsaicin 20 and capsaicin 50 groups compared with untreated adipocytes (p<0.001) and significantly decreased in the capsaicin 0.5 group (p<0.05, Figure 6).

NF-kB1 levels were significantly decreased in lower doses of capsaicin (0.5μM and 1μM) capsaicin-treated adipocytes and significantly increased in the highest dose of capsaicin (50μM) capsaicin-treated adipocytes, in comparison with untreated adipocytes (p<0.001, Figure 6).

Impact of Capsaicin on TRPV-1, UCP-2, and SIRT-1 Expression

Except for capsaicin 20, significant decreases in TRPV-1 levels were observed in all capsaicin groups compared with untreated adipocytes (p<0.001). Conversely, in the capsaicin 20 group, TRPV-1 levels significantly increased (p<0.001, Figure 7).

Additionally, UCP-2 levels exhibited significant decreases in all capsaicin groups (p<0.001). Similarly, SIRT-1 levels were decreased significantly in all Capsaicin groups, except for capsaicin 20, when compared with untreated adipocytes (p<0.001). Notably, SIRT-1 levels were significantly increased in the capsaicin 20 group (p<0.001, Figure 7).

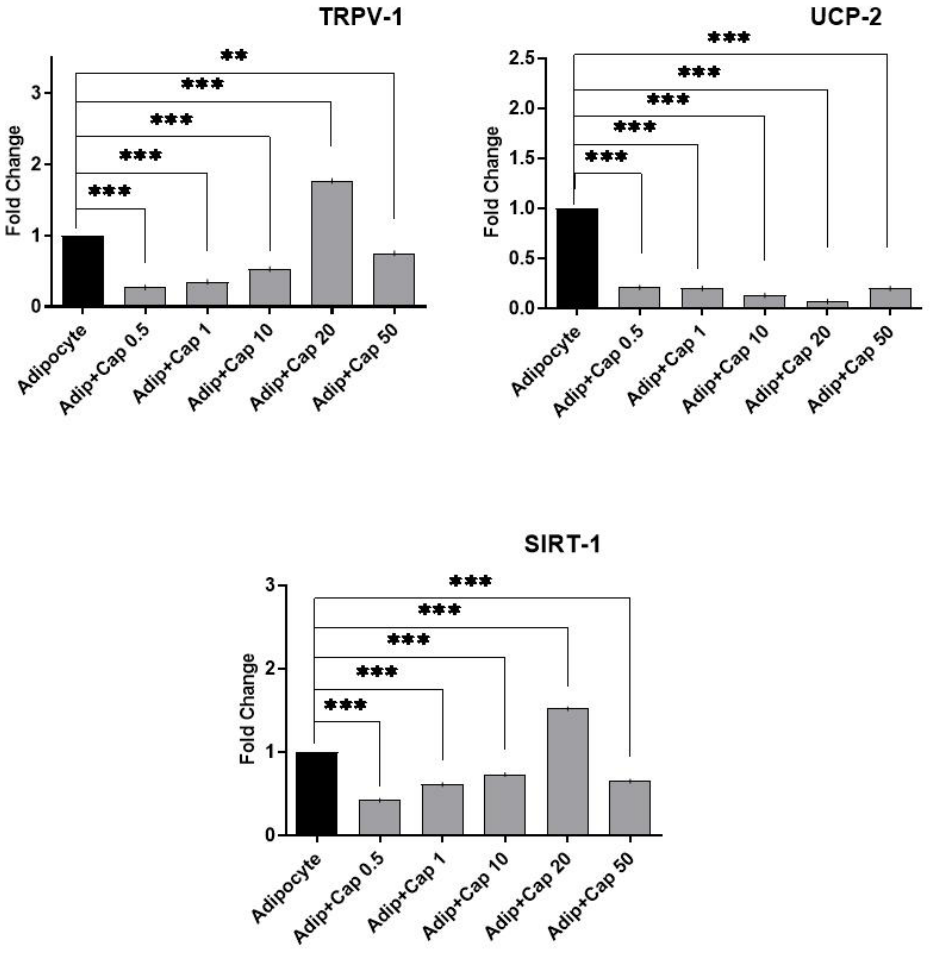

Figure 7. Effects of different doses of capsaicin treatment on TRPV-1, UCP-2, and SIRT-1 gene expression levels. **p<0.01; ***p<0.001; Cap: Capsaisin; Adip: Adipocyte.

DISCUSSION

It is well known that weight loss is very effective in reducing the complications of obesity-related disorders. For this purpose, many studies have focused on the investigation of food supplements that suppress body fat storage. Recent studies have shown that many natural food components have beneficial effects on weight loss and can prevent obesity (18, 19). One of them, capsaicin, was reported to be effective in reducing body weight and fat storage in vivo and suppressing adipogenesis in vitro (9, 20-22).

As adipose tissue is an active source of inflammation, illumination of the effect of capsaicin on adipocyte differentiation is of great importance. In this study, we demonstrated that adipocyte differentiation and adipose tissue-related inflammatory protein expression were suppressed by dose-dependent capsaicin supplementation.

The main role of adipose tissue is to store fat for energy uptake (23). There are two types of adipose tissue: the first is WAT which stores lipids and oxidizes them when energy is required. The second type is BAT, which is rich in mitochondria and burns lipids to generate heat by activating TRPV1 (23). Capsaicin induces TRPV1-mediated Ca^{2+} influx and reduces adipogenesis and obesity. Zhang et al. reported that long-term feeding with capsaicin prevented fat storage in WAT, whereas TRPV1 null-mice feeding with a high-fat diet + capsaicin was not effective on adipogenesis and obesity (21). Baskaran et al. demonstrated that capsaicin stimulates SIRT1-dependent deacetylation of PPARγ and induces BAT activation (23). It has been reported that capsaicin has generated WAT browning by inducing SIRT-dependent deacetylation by TRPV1 activation (7). Our results are consistent with those of previous studies. Capsaicin has stimulated the protein expressions of SIRT1 and TRPV-1 in adipocytes. According to our findings, the most effective capsaicin dosage for the regulation of SIRT1 and TRPV-1 expressions seems to be 20 µM.

The transcription factors PPARγ and C/EBP-α are known key proteins in the regulation of adipogenic differentiation (24). Ibrahim et al. have shown that capsaicin significantly reduced the mRNA expression of PPARγ and C/EBP-α (25). A recent study has reported that capsaicin decreased the PPARγ, C/EBP-α, and leptin expression and inhibited preadipocyte differentiation (9). In this study, capsaicin supplementation suppressed PPARγ, C/EBP-α, and leptin expression. According to our findings, capsaicin appears to effectively reduce PPARγ and C/EBP-α expressions at lower and higher concentrations.

Leptin expressions were found to be decreased at 0.5 μM and 1 μM capsaicin concentrations in this study. The decreased expression of leptin in a dose-dependent manner by capsaicin is a remarkable finding for its anti-lipogenic ability, especially at lower doses of capsaicin. The upper doses (10, 20, and 50 μM) have seemed to stimulate leptin expression and induce an inflammatory response. According to our findings, capsaicin reduced adiponectin expression. Babbota et al. reported that adiponectin secretion remained unchanged in adipocytes supplemented with capsaicin (16). Recent studies have reported contradictory results regarding adiponectin secretion. Several studies have reported its pro-inflammatory effects (26, 27), while others have supported its anti-inflammatory effects (28, 29). In this study, adiponectin seems to act as a pro-inflammatory mediator, and its expression was decreased with capsaicin supplementation.

Another important point is to focus on the response of NF-κB, a major transcription factor in inflammation, to capsaicin during adipocyte differentiation. Kang et al. have shown that capsaicin inhibits NF-κB activation in adipocytes (8). In the present study, NF-κB expression and inflammatory adipokines, such as IL-6 and TNF-α reduced by capsaicin supplementation. Consistent with these results, cyclooxygenase-2 (COX-2), which is an inflammatory marker, was significantly decreased in a dose-independent manner. NF-κB and IL-6 seem to be sensitive to lower doses of capsaicin, but TNF-α expression was reduced by 0.5 μM capsaicin, whereas the opposite effect was observed at 20 and 50 μM doses. Our findings are consistent with those by Baboota et al. (16). The inhibitory effect of capsaicin on the inflammatory state appears to be effective at lower capsaicin doses (0.5-1 μM).

UCP2, which controls energy expenditure, is highly expressed in human white adipose tissue (30). UCP2 is a mitochondrial proton transporter that influences thermogenesis and is related to lipid accumulation (31). Oliveira et al. have shown that UCP2 gene expression is decreased in patients with obesity (32). Heinitz et al. reported significant downregulation of UCP2 in the skeletal muscle of patients with obesity (33). In contrast, Vidal-Puig et al. detected that UCP2 expression was not altered in the adipose tissues of obese and lean individuals (34). According to our current knowledge, there is a single recent study that has evaluated the association between UCP2 and capsaicin in patients with obesity. Lee et al. reported that UCP2 expression was increased with 0.1, 1, and 10 μM capsaicin doses in cultured adipocyte cells (35). They have informed that UCP2 is related to mitochondrial antioxidant mechanisms and controls reactive oxygen species (ROS) production (35, 36). In another study, Ding et al. demonstrated that UCP2 ameliorated mitochondrial dysfunction by reducing the inflammatory state and oxidative stress in an experimental acute kidney injury model. UCP2 decreased cytokine release and inhibited NF-κB activation (37). In previous studies, UCP2 was shown to reduce adipogenesis and regulate genes related to lipid metabolism (9, 35). It has been reported to exert anti-obesity effects by activating the TRPV1 receptor. However, a previous study has shown that its anti-inflammatory effect was independent of TRPV1 (8). In the present study, in contrast to the results reported by Lee et al., UCP2 expression was decreased in the presence of capsaicin. The regulation of UCP2 expression with capsaicin is still not completely understood. According to the findings of our study, the regulatory anti-inflammatory mechanism of capsaicin may be completely independent of its adipogenesis-regulating mechanism.

These findings indicate that capsaicin exerts an inhibitory influence on adipocyte differentiation. This finding is consistent with that of a previous study that reported the anti-inflammatory effect of capsaicin through NF-κB inactivation (8). However, the inhibitory effects of capsaicin on cytokine secretion and UCP2 expression appear to be more complex and involve different pathways. According to our study, it has been concluded that although the effect of capsaicin on proteins regulating adipogenesis is not dose-related, however, its inhibitory effect on adiposity-dependent inflammation was more pronounced at low doses. We would like to point out that this study also has some limitations. One important point is that protein expression levels cannot be determined. Furthermore, we could not measure triglyceride levels, which is another indicator of lipid levels, along with oil red o staining. However, this study is important because it is the first to investigate the effects of capsaicin on human adipocyte cells.

Ethics Committee Approval: For this type of study formal ethical consent is not required.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- S.D., P.C., M.S., S.T.K., H.K., Y.O.I.; Data Acquisition: S.D., P.C., S.T.K.; Data Analysis/Interpretation: M.S., Y.O.I., H.K.; Drafting Manuscript- S.D., Y.O.I.; Critical Revision of Manuscript- S.D., P.C., M.S., S.T.K., H.K., Y.O.I.; Final Approval and Accountability- S.D., P.C., M.S., S.T.K., H.K., Y.O.I.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was supported by the Scientific Research Projects Unit of Demiroğlu Bilim University (project number: 2016/01-06).

REFERENCES

1. Ntambi JM, Young-Cheul K. Adipocyte differentiation and gene expression. J Nutr 2000; 130(12): 3122S-26S.
2. Varghese S, Kubatka P, Rodrigo L, Gazdikova K, Caprnda M, Fedotova J, et al. Chili pepper as a body weight-loss food. Int J Food Sci Nutr 2017; 68(4): 392-401.
3. Whiting S, Derbyshire E, Tiwari BK. Capsaicinoids and capsinoids. A potential role for weight management? A systematic review of the evidence. Appetite 2012; 59(2): 341-8.
4. Chen J, Li L, Li Y, Liang X, Sun Q, Yu H, et al. Activation of TRPV1 channel by dietary capsaicin improves visceral fat remodeling through connexin43-mediated $Ca2^{+}$ influx. Cardiovasc Diabetol 2015; 14:22.
5. Sun F, Xiong S, Zhu Z. Dietary capsaicin protects cardiometabolic organs from dysfunction. Nutrients 2016; 8(5): 174.
6. Zheng J, Zheng S, Feng Q, Zhang Q, Xiao X. Dietary capsaicin and its anti-obesity potency: from mechanism to clinical implications. Biosci Rep 2017; 37(3): BSR20170286.
7. Baskaran P, Krishnan V, Ren J, Thyagarajan B. Capsaicin induces browning of white adipose tissue and counters obesity by activating TRPV1 channel-dependent mechanisms. Br J Pharmacol 2016; 173(15): 2369-89.
8. Kang JH, Kim CS, Han IS, Kawada T, Yu R. Capsaicin, a spicy component of hot peppers, modulates adipokine gene expression and protein release from obese-mouse adipose tissues and isolated adipocytes, and suppresses the inflammatory responses of adipose tissue macrophages. FEBS Lett 2007; 581(23): 4389-96.
9. Hsu CL, Yen GC. Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. J Agric Food Chem 2007; 55(5): 1730-6.
10. Mahadik SR, Lele RD, Saranath D, Seth A, Parikh V. Uncoupling protein-2 (UCP2) gene expression in subcutaneous and omental adipose tissue of Asian Indians: Relationship to adiponectin and parameters of metabolic syndrome. Adipocyte 2012; 1(2): 101-7.
11. Oberkofler H, Liu YM, Esterbauer H, Hell E, Krempler F, Patsch W. Uncoupling protein-2 gene: reduced mRNA expression in intraperitoneal adipose tissue of obese humans. Diabetologia 1998; 41(8): 940-6.
12. Zamora-Mendoza R, Rosas-Vargas H, Ramos-Cervantes MT, Garcia-Zuniga P, Perez-Lorenzana H, Mendoza-Lorenzo P, et al. Dysregulation of mitochondrial function and biogenesis modulators in adipose tissue of obese children. Int J Obes (Lond) 2018; 42(4): 618-24.
13. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders. Nature 2017; 542(7640): 177-85.
14. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. J Clin Invest 2011; 121(6): 2111-7.
15. Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. J Endocrinol 2014; 222(3): R113-27.
16. Baboota RK, Singh DP, Sarma SM, Kaur J, Sandhir R, Boparai RK, et al. Capsaicin induces "brite" phenotype in differentiating 3T3-L1 preadipocytes. PLoS One 2014; 9: 7.
17. Pinar C, Degirmencioglu S, Tanrikulu Kucuk S, Seyithanoglu M, Oner Iyidogan Y, Kocak H. Association of different doses of curcumin with preadipocyte-adipocyte differentiation. Med J Bakirkoy 2024; (in press).
18. Lee MS, Kim CT, Kim IH, Kim Y. Inhibitory effects of green tea catechin on the lipid accumulation in 3T3-L1 adipocytes. Phytother Res 2009; 23(8): 1088-91.
19. Lee MS, Kim CT, Kim Y. Green tea (-)-epigallocatechin-3-gallate reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. Ann Nutr Metab 2009; 54(2): 151-7.
20. Inoue N, Matsunaga Y, Satoh H, Takahashi M. Enhanced energy expenditure and fat oxidation in humans with high BMI scores by the ingestion of novel and non-pungent capsaicin analogues (capsinoids). Biosci Biotechnol Biochem 2007; 71(2): 380-9.
21. Zhang LL, Yan Liu D, Ma LQ, Luo ZD, Cao TB, Zhong J, et al. Activation of transient receptor potential vanilloid type-1 channel prevents adipogenesis and obesity. Circ Res 2007; 100(7): 1063-70.
22. Kang SI, Kim MH, Shin HS, Kim HM, Hong YS, Park JG, et al. A water-soluble extract of Petalonia binghamiae inhibits the expression of adipogenic regulators in 3T3-L1 preadipocytes and reduces adiposity and weight gain in rats fed a high-fat diet. J Nutr Biochem 2010; 21(12): 1251-7.
23. Cinti S. Transdifferentiation properties of adipocytes in the adipose organ. Am J Physiol Endocrinol Metab 2009; 297(5): E977-86.
24. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes Dev 2000; 14(11): 1293-307.
25. Ibrahim M, Jang M, Park M, Gobianand K, You S, Yeon SH, et al. Capsaicin inhibits the adipogenic differentiation of bone marrow mesenchymal stem cells by regulating cell proliferation, apoptosis, oxidative and nitrosative stress. Food Funct 2015; 6(7): 2165-78.
26. Lappas M, Permezel M, Rice GE. Leptin and adiponectin stimulate the release of proinflammatory cytokines and prostaglandins from human placenta and maternal adipose tissue via nuclear factor-kappaB, peroxisomal proliferator-activated receptor-gamma and extracellularly regulated kinase 1/2. Endocrinology 2005; 146(8): 3334-42.
27. Cheng X, Folco EJ, Shimizu K, Libby P. Adiponectin induces pro-inflammatory programs in human macrophages and CD4+ T cells. J Biol Chem 2012; 287(44): 36896-904.
28. Lira FS, Rosa JC, Pimentel GD, Seelaender M, Damaso AR, Oyama LM. Both adiponectin and interleukin-10 inhibit LPS-induced activation of the NF-κB pathway in 3T3-L1 adipocytes. Cytokine. 2012; 57(1): 98-106.
29. Ajuwon KM, Spurlock ME. Adiponectin inhibits LPS-induced NF-kappaB activation and IL-6 production and increases PPARgamma2 expression in adipocytes. Am J Physiol Regul Integr Comp Physiol 2005; 288(5): R1220-5.
30. Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, et al. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet 1997; 15(3): 269-72.
31. Rousset S, Alves-Guerra MC, Mozo J, Miroux B, Cassard-Doulcier AM, Bouillaud F, et al. The biology of mitochondrial uncoupling proteins. Diabetes 2004; 53 Suppl 1: S130-5.
32. Oliveira MS, Rheinheimer J, Moehlecke M, Rodrigues M, Assmann TS, Leitão CB, et al. CP2, IL18, and miR-133a-3p are dysregulated in subcutaneous adipose tissue of patients with obesity. Mol Cell Endocrinol 2020; 509: 110805.
33. Heinitz S, Piaggi P, Yang S, Bonfiglio S, Steel J, Krakoff J, et al. Response of skeletal muscle UCP2-expression during metabolic adaptation to caloric restriction. Int J Obes (Lond) 2018; 42(5): 974-84.
34. Vidal-Puig A, Rosenbaum M, Considine RC, Leibel RL, Dohm GL, Lowell BB. Effects of obesity and stable weight reduction on UCP2 and UCP3 gene expression in humans. Obes Res 1999; 7(2): 133-40.
35. Lee MS, Kim CT, Kim IH, Kim Y. Effects of capsaicin on lipid catabolism in 3T3-L1 adipocytes. Phytother Res 2011; 25(6): 935-9.

36. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 2005; 70(2): 200-14.

37. Ding Y, Zheng Y, Huang J, Peng W, Chen X, Kang X, et al. UCP2 ameliorates mitochondrial dysfunction, inflammation, and oxidative stress in lipopolysaccharide-induced acute kidney injury. Int Immunopharmacol 2019; 71: 336-49.

Experimed 2024; 14(2): 126-130

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1494644

Apelin Levels According to the Site of Involvement in Inflammatory Bowel Diseases

Ezgi Degerli[1], Selcan Cesur[2], Ahmet Yavuz[3], Mahmut Said Degerli[4], Zeynep Ermis Karaali[5]

[1]Department of Medical Oncology, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, University of Health Sciences, Istanbul, Turkiye
[2]Department of Gastroenterology, Eskisehir City Hospital, Eskisehir, Turkiye
[3]Department of Gastroenterology, Diyarbakir Gazi Yasargil Training and Research Hospital, Diyarbakir, Turkiye
[4]Department of General Surgery, Bakirkoy Dr. Sadi Konuk Training and Research Hospital, University of Health Sciences, Istanbul, Turkiye
[5]Department of Internal Medicine, Basaksehir Cam and Sakura City Hospital, Istanbul, Turkiye

ORCID ID: E.D. 0000-0002-8664-5701; S.C. 0000-0002-1504-7069; A.Y. 0000-0002-4633-5012; M.S.D. 0000-0002-8313-7904; Z.E.K. 0000-0002-3770-287X

Cite this article as: Degerli E, Cesur S, Yavuz A, Degerli MS, Ermis Karaali Z. Apelin levels according to the site of involvement in inflammatory bowel diseases. Experimed 2024; 14(2): 126-130.

ABSTRACT

Objective: Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), presents ongoing challenges in terms of diagnosis and management. With increasing treatment options, reliable markers for assessing treatment response have become crucial. This study explored the potential of apelin, a peptide hormone implicated in inflammation, as a biomarker for monitoring disease activity in patients with IBD undergoing colonoscopy.

Materials and Methods: The study included 91 patients who were followed up for IBD. Apelin levels were measured in serum, and the site of involvement was simultaneously examined by colonoscopy. Alternations of apelin levels depending on the location of involvement was also considered.

Results: Despite the widespread presence of apelin in the gastrointestinal system (GIS), our findings did not reveal significant differences in apelin levels among patients with varying colonoscopic involvement (p=0.73).

Conclusion: Although apelin has potential as a biomarker of gastrointestinal inflammation in IBD, its precise role and clinical applicability necessitate comprehensive studies involving larger patient populations. Future research on apelin and IBD could refine its utility in disease monitoring and enhance its diagnostic significance.

Keywords: Inflammatory bowel disease, apelin, Crohn's disease, ulcerative colitis

INTRODUCTION

Inflammatory bowel disease (IBD) has two chronic forms. Ulcerative colitis (UC) is a chronic inflammatory bowel disease characterized by remissions and exacerbations that involve the colonic mucosa without leaving intact tissue through various extensions from the rectum to the proximal. Crohn's disease (CD), in turn, affects the entire digestive tract from the mouth to the anus in a segmental and transmural manner, and it also presents with remissions and exacerbations; however, it should be known that both conditions are not specific to the digestive tract, but are systemic diseases with many extra intestinal symptoms. Factors such as genetic, environmental, and host immune responses are believed to be responsible for the etiology of these conditions. IBD has a complex diagnosis, which is based on clinical, endoscopic, and histological features (1, 2).

The treatment of both diseases is based on immunosuppressive therapy. In recent years, immunomodulatory therapies have been at the forefront. The increasing availability of new treatment options reduces morbidity among these patients. Alongside the expansion of treatment choices, numerous immunological

Corresponding Author: Ezgi Degerli **E-mail:** ezgitastan.19@gmail.com
Submitted: 03.06.2024 **Revision Requested:** 16.07.2024 **Last Revision Received:** 03.08.2024 **Accepted:** 05.08.2024

markers used in monitoring have become crucial. The desirability of a noninvasive method for assessing treatment response is highly valuable (3).

In this context, biochemical markers have become increasingly popular in recent years. One pertinent marker is apelin, whose levels increase during inflammation and can be measured using a simple blood test. Apelin is a peptide hormone recently added to the adipokine family that is released from adipose tissue. It was first isolated from bovine gastric juice (4). Apelin and apelin receptor ligands have been identified in many tissues throughout the body. It is primarily present in the human body's gastric epithelium and myocardial tissue (5). Most studies on apelin focused on the cardiovascular system. This is because apelin is present in the entire endothelium, including arteries, veins, and small vessels. It has increased nitric oxide release and to have positive inotropic effects on the heart (6).

Apelin also plays an active role in the gastrointestinal system (GIS). Apelin receptors are commonly found in the GIS (gastric enterochromaffin, pancreatic, colonic epithelial cells, gastric fundus, duodenum, and ileum). It stimulates cholecystokinin secretion in the intestinal tract and increases gastric cell proliferation. In addition, it blocks histamine release from enterochromaffin cells of the stomach and causes less acid release from parietal cells (7, 8). It exerts an inhibitory effect on dose-dependent gastric emptying and intestinal transit.

Furthermore, examining the gastrointestinal effects of apelin led to the discovery of its effects on food intake. Centrally administered apelin decreased food intake, whereas intravenous apelin administration did not affect food intake. However, studies on this subject should be more consistent. This distinction has led to the thought that individual differences may be related to apelin (9).

The widespread presence of apelin in the GIS prompted us to consider whether its expression undergoes alterations during chronic inflammation. This study examined apelin levels in the peripheral blood of patients with IBD who exhibited segmental or total active disease during colonoscopy. We hypothesized that individuals with widespread colonoscopic disease might exhibit higher levels of apelin than those with borderline disease. Consequently, we assessed changes in the inflammatory involvement in this disease, which often requires frequent colonoscopy monitoring.

MATERIALS AND METHODS

The Code of Ethics of the World Medical Association (Declaration of Helsinki) was used for the experiments involving human subjects. All procedures were performed in compliance with relevant laws and institutional guidelines. The Non-Drug Clinical Research Ethics Committee of Haseki Training and Research Hospital obtained the study's ethical approval (date: 05.04.2017, IRB number: 476).

The study included patients diagnosed with IBD who were followed up at the Gastroenterology Clinic of Training and Research Hospital after obtaining written informed consent. Patients younger than 65 years who were diagnosed with IBD endoscopically and histopathologically were included in the

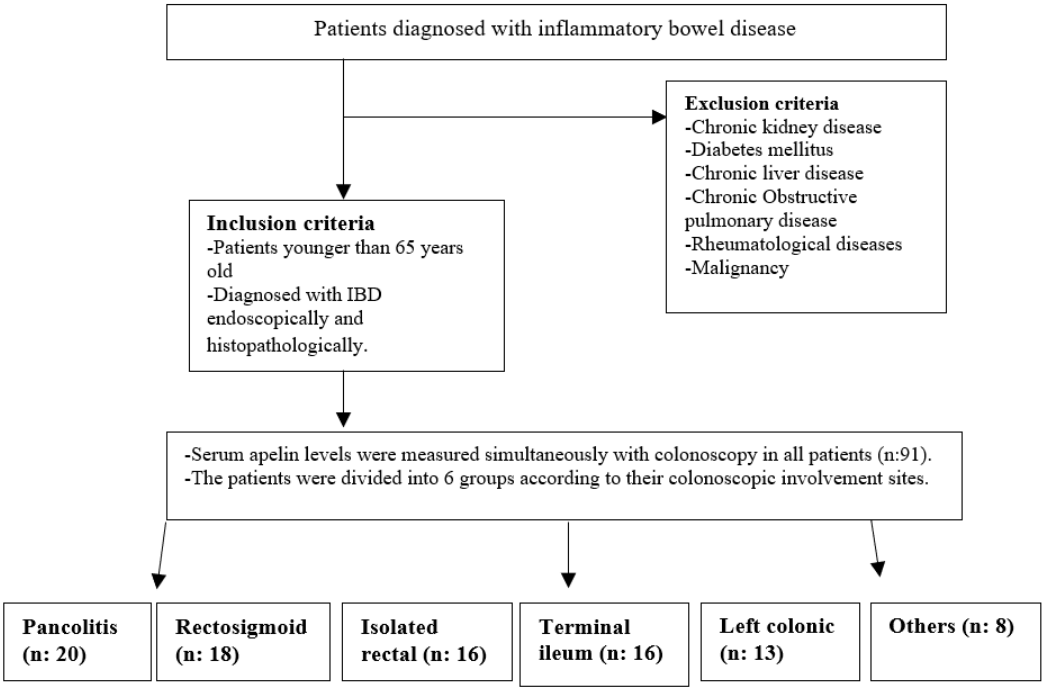

Figure 1. Patient flow chart. IBD, Inflammatory bowel disease.

study. Patients were divided into six groups according to their colonoscopic involvement sites (Figure 1).

The exclusion criteria were the presence of chronic kidney disease, diabetes mellitus, chronic liver disease, chronic obstructive pulmonary disease, rheumatologic diseases, and malignancies.

Biochemical Analysis

Samples were collected from venous blood and placed into gel tubes for apelin analysis. Within half an hour, the samples were centrifuged at 1000xg for 10 min and stored at -80°C. Serum apelin levels were detected using the Immulite 200 device (SIEMENS) with the CLIA (chemiluminescence immunoassay) method in all study patients. The site of involvement was demonstrated macroscopically and histopathologically by colonoscopy.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS) 15 statistical software (SPSS Inc, Chicago, IL, USA) for windows was used. Descriptive statistics were expressed as numbers and percentages for categorical variables, whereas the mean, standard deviation, and median were used for numerical variables. When the average distribution assumptions were unmet, a Kruskal-Wallis test was used to compare numerical variables between multiple groups. The alpha level of statistical significance was set as $p<0.05$.

RESULTS

The total number of patients with IBD was 91, consisting of 67 patients with UC and 24 patients with CD. The patients diagnosed with UC included 37 females and 30 males. Patients diagnosed with CD included 11 females and 13 males. The mean ages of patients with UC and CD were 38 and 31 years, respectively. The disease duration ranged from 4 to 22 years among patients with UC and from 1 to 13 years among patients with CD. The distribution of patients with inflammatory bowel disease by site of involvement was examined. Accordingly, 20 patients had pancolitis, 18 had recto-sigmoid arthritis, 16 had isolated rectal arthritis, 16 had terminal ileal arthritis, 13 had left colonic and 8 with others involvement (1 with transverse colonic, 2 with right colonic, 4 with caecal, and 1 with perianal).

Apelin's levels were analyzed by gender in IBD patients. The values were 1457.4 ± 1021.7 pg/mL in male patients and 1765.4 ± 1320.6 pg/mL in female patients. There were no significant differences in apelin levels between genders (p=0.323).

The apelin levels were compared with inflammatory markers routinely used during the follow-up of IBD. No significant association was found between C-reactive protein (CRP), sedimentation, hemoglobin, white blood cell (WBC), and apelin levels (Table 1).

Table 1. Comparison of apelin levels with other inflammatory markers and hemoglobin levels in patients with inflammatory bowel disease.

	Apelin Level (pg/mL)	
	rho	**p-value**
CRP	-0.132	0.231
Sedimentation	-0.085	0.440
Hemoglobin	0.036	0.745
Hematocrit	-0.012	0.917
WBC	-0.014	0.896

CRP, C-reactive protein; WBC, white blood cell.

Table 2. Comparison of apelin levels in inflammatory bowel disease according to site of involvement.

Site of Involvement	**n**	**Apelin level (pg/mL)**		
		Mean	**SD**	**Median**
Terminal Ileum	16	1522.6	1092.9	1154.0
Left Colon	13	1461.9	1238.7	841.7
Rectum	16	1645.5	1338.8	1012.0
Rectosigmoid	18	1661.8	1134.9	1231.2
Pancolitis	20	1843.1	1276.0	1560.0
Others	8	943.26	312.0	920.0

There were no significant differences in apelin levels between groups (p=0.730). n, number of samples; SD, Standard deviation.

This study compared the apelin levels of patients with IBD according to the site of involvement. Apelin levels did not differ significantly between patients divided into six group involvement sites on colonoscopy (p=0.730; Table 2).

DISCUSSION

Several inflammatory markers and biomarkers have been examined during follow-up of patients with IBD. Although some markers have contributed to assess the presence or absence of inflammation, most have been included in treatment follow-up. Especially fecal calprotectin, which has recently come into use, provides valuable information as a non-invasive inflammatory marker. In this regard, a study by Mosli et al. demonstrated that it was quite significant to examine fecal calprotectin in diagnosing patients with IBD (10, 11). Today, fecal calprotectin can also be used to identify new cases with suspected diagnosis. In addition, it can be examined regularly in patients diagnosed and followed up and provides beneficial results in remission activation (12). However, fecal calprotectin does not register elevated levels in diseases

characterized by ileal involvement. In other words, although it serves as a marker applicable to IBD with colonic involvement, the disease may be active in cases of ileal involvement. At the same time, fecal calprotectin levels remained within the normal range. Similar to apelin, fecal calprotectin cannot predict the extent of segmental involvement in colonic disease.

Apelin levels increase during inflammation and may elevate secondary responses arising from this inflammation. Lu et al. demonstrated a significant elevation in apelin levels in secondary cardiac hypertrophy induced by stress (13). Consistently, increased levels of angiotensin 2 were found in cardiac hypertrophy. This observation suggests the potential utility of apelin as a cardiac marker in the context of cardiac hypertrophy. Topuz et al. also demonstrated elevated apelin levels associated with cardiac stress, including cardiomyopathies (14).

The most compelling findings in cardiovascular studies are from data on patients with myocardial ischemia. Tycinska et al. observed elevated apelin levels in non-ST elevation myocardial infarction. Furthermore, the authors noted a significant association between high apelin levels and mortality within the first six months (15).

Apelin tends to increase even in chronic, slowly progressing inflammatory processes. Zarei et al. identified elevated levels of apelin and demonstrated that the levels of apelin released from adipose tissue are not influenced by dietary anti-oxidant levels (16).

Continuous inflammation followed by fibrosis during healing may contribute to elevated apelin levels. Kocer et al. observed elevated levels of apelin in renal interstitial fibrosis (17). Moreover, similar to our study, the authors demonstrated that this elevation was unrelated to CRP.

Experimental studies have shown a relationship between serum apelin levels and inflammatory bowel disease. These studies have shown that apelin levels are associated with trans mucosal inflammation of the colonic epithelium, stenosis, fibrosis, and mucosal healing. Song Han et al. (18) have also provided an essential guide for GIS. IBD-related studies include experimental investigations conducted by Song Han and colleagues. Experimentally inducing colitis and inflammatory bowel syndrome, the authors found elevated levels of apelin. In this study, experimental colitis was induced, and it was observed that exogenously administered apelin increased colonic proliferation (19-21). Additionally, it was noted that this marker released from the colonic epithelium could regulate epithelial proliferation. Consequently, the authors suggested that apelin plays a role in multiple stages of colonic differentiation. This proposition implies that apelin levels may vary among colon segments during inflammation.

A recent study by Mantaka et al. (22) compared the serum apelin levels in healthy individuals and patients diagnosed with IBD and found that the apelin level was significantly higher in patients diagnosed with IBD (p=0.012). The serum apelin level was found to be similar to that in our study. In this study, no distinction was made according to the location of involvement. However, no significant difference was found in the distinction according to the severity of endoscopic involvement (moderate-severe). The presence of a control group in the study by Mantaka et al. is an advantage of the study. The inclusion and exclusion criteria of patients, as well as the small sample size, are similar to those in our study. Mantaka et al. stated that the small number of patients was due to the COVID-19 pandemic (22).

In our study, we identified active sites of disease involvement using colonoscopy. During the same period, we investigated whether peripheral blood apelin levels differed according to the location of involvement. As anticipated, patients with active colonic involvement exhibited higher apelin levels. Although numerically higher in patients with pancolitis, no statistically significant elevation could be established, potentially due to our study's limited number of patients and samples. The lower incidence of pancolitis might be attributed to the presence of severe clinical symptoms and declining participation in the study. The most important limitation of this study is the small number of patients.

In conclusion, although apelin has been shown to be significant in various inflammatory conditions, its specific role in IBD remains complex and warrants further investigation. Prospective studies with a larger patient population focusing on apelin and IBD could provide more comprehensive insights into its potential as a biomarker for disease activity.

Ethics Committee Approval: Approval was received from the Non-Drug Clinical Research Ethics Committee of Haseki Training and Research Hospital (date: 05.04.2017, IRB number: 476).

Informed Consent: Informed written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.D., S.C.; Data Acquisition: S.C., A.Y.; Data Analysis/Interpretation: M.S.D., E.K.; Drafting Manuscript- E.D., S.C., A.Y.; Critical Revision of Manuscript- M.S.D., Z.E.K.; Final Approval and Accountability- E.D., S.C., A.Y., M.S.D., Z.E.K.

Conflicts of Interests: The authors declare that they have no competing interests.

Financial Disclosure: This study was supported by the Haseki Training and Research Hospital Management Thesis and Academic Studies Advisory and Monitoring Commission (TACDIK).

REFERENCES

1. Kofla-Dłubacz A, Pytrus T, Akutko K, Sputa-Grzegrzółka P, Piotrowska A, Dzięgiel P. Etiology of IBD-is it still a mystery? Int J Mol Sci 2022; 23(20): 12445.
2. Jarmakiewicz-Czaja S, Zielińska M, Sokal A, Filip R. Genetic and epigenetic etiology of ınflammatory bowel disease: an update. Genes (Basel) 2022; 13(12): 2388.
3. Seyedian SS, Nokhostin F, Malamir MD. A review of the diagnosis, prevention, and treatment methods of inflammatory bowel disease. J Med Life 2019; 12(2): 113-22.
4. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 1998; 251(2): 471-6.
5. Földes G, Horkay F, Szokodi I, Vuolteenaho O, Ilves M, Lindstedt KA, et al. Circulating and cardiac levels of apelin, the novel ligand of the orphan receptor APJ, in patients with heart failure. Biochem Biophys Res Commun 2003; 308(3): 480-5.
6. Japp AG, Newby DE. The apelin-APJ system in heart failure: pathophysiologic relevance and therapeutic potential. Biochem Pharmacol 2008; 75(10): 1882-92.
7. Susaki E, Wang G, Cao G, Wang HQ, Englander EW, Greeley GH Jr. Apelin cells in the rat stomach. Regul Pept 2005; 129(1-3): 37-41.
8. Wang G, Anini Y, Wei W, Qi X, OCarroll AM, Mochizuki T, et al. Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. Endocrinology 2004; 145(3): 1342-8.
9. Pitkin SL, Maguire JJ, Bonner TI, Davenport AP. International Union of Basic and Clinical Pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology, and function. Pharmacol Rev 2010; 62(3): 331-42.
10. Mosli MH, Zou G, Garg SK, Feagan SG, MacDonald JK, Chande N, et al. C-reactive protein, fecal calprotectin, and stool lactoferrin for detection of endoscopic activity in symptomatic inflammatory bowel disease patients: a systematic review and meta-analysis. Am J Gastroenterol 2015; 110(6): 802-20.
11. Sipponen T, Kolho KL. Fecal calprotectin in diagnosis and clinical assessment of inflammatory bowel disease. Scand J Gastroenterol 2015; 50(1): 74-80.
12. Guardiola J, Lobatón T, Rodríguez-Alonso L, Ruiz-Cerulla A, Arajol C, Loayza C, et al. Fecal level of calprotectin identifies histologic inflammation in patients with ulcerative colitis in clinical and endoscopic remission. Clin Gastroenterol Hepatol 2014; 12(11): 1865-70.
13. Lu L, Wu D, Li L, Chen L. Apelin/APJ system: A bifunctional target for cardiac hypertrophy. Int J Cardiol 2017; 230: 164-70.
14. Topuz M, Cosgun M, Akkuş O, Bulut A, Sen O, Topuz AN, et al. Effect of spironolactone on plasma Apelin-12 levels in patients with chronic systolic heart failure. Acta Cardiol Sin 2016; 32(6): 690-7.
15. Tycinska AM, Lisowska A, Musial WJ, Sobkowicz B. Apelin in acute myocardial infarction and heart failure induced by ischemia. Clin Chim Acta 2012; 413(3-4): 406-10.
16. Zarei S, Saidijam M, Karimi J, Yadegarazari R, Rezaei Farimani A, Hosseini-Zijoud SS, et al. Effect of resveratrol on resistin and apelin gene expressions in adipose tissue of diabetic rats. Turk J Med Sci 2016; 46(5): 1561-7.
17. Kocer D, Karakukcu C, Ozturk F, Eroglu E, Kocyigit I. Evaluation of fibrosis markers: apelin and transforming growth factor-β1 in autosomal dominant polycystic kidney disease patients. Ther Apher Dial 2016; 20(5): 517-22.
18. Han S, Wang G, Qiu S, de la Motte C, Wang HQ, Gomez G, et al. Increased colonic apelin production in rodents with experimental colitis and in humans with IBD. Regul Pept 2007; 142(3): 131-7.
19. Ge Y, Li Y, Chen Q, et al. Adipokine apelin ameliorates chronic colitis in Il-10$^{-/-}$ mice by promoting intestinal lymphatic functions. Biochem Pharmacol 2018; 148: 202-12.
20. Desreumaux P, Ernst O, Geboes K, Gambiez L, Berrebi D, Müller-Alouf H, et al. Inflammatory alterations in mesenteric adipose tissue in Crohn's disease. Gastroenterology 1999; 117: 73–81.
21. Guedj K, Abitbol Y, Cazals-Hatem D, Morvan M, Maggiori L, Panis Y, et al. Adipocytes orchestrate the formation of tertiary lymphoid organs in the creeping fat of Crohn's disease affected mesentery. J Autoimmun 2019; 103: 102281.
22. Mantaka A, Kalyvianaki K, Kastritsi O, Kampa M, Koutroubakis IE. Increased serum apelin levels in patients with inflammatory bowel disease. Gastroenterology Insights 2024; 15(2): 255-65.

EXPERIMED

AIMS AND SCOPE

Experimed is a scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. It is the official online-only publication of İstanbul University Aziz Sancar Institute of Experimental Medicine and published three times a year in April, August, and December.

The journal targets national and international audiences, has both national and international members in the editorial board, and aims to enhance international author contributions.

As of 2022 the publication language of the journal is only English. The manuscripts submitted for publication in the journal must be scientific work in English.

Experimed aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of basic and clinical medical sciences. The journal publishes original articles, case reports, reviews, and letters to the editor that are prepared in accordance with ethical guidelines.

The scope of the journal includes but not limited to; experimental studies in all fields of medical sciences.

The target audience of the journal includes specialists and professionals working and interested in all disciplines of basic and clinical medical sciences.

There are no article processing charges or submission fees for any submitted or accepted articles. All expenses of the journal are covered by the Istanbul University.

Statements or opinions expressed in the manuscripts published in the journal reflect the views of the author(s) and not the opinions of the İstanbul University Aziz Sancar Institute of Experimental Medicine, editors, editorial board, and/or publisher; the editors, editorial board, and publisher disclaim any responsibility or liability for such materials.

Editor in Chief: Prof. Dr. Bedia Cakmakoglu
Address: Istanbul University, Aziz Sancar Institute of Experimental Medicine, Vakıf Gureba Avenue, 34093, Capa, Fatih, Istanbul, Turkiye
Phone: +90 212 414 2000-33305
Fax: +90 212 532 4171
E-mail: bedia@istanbul.edu.tr

Publisher: Istanbul University Press
Address: Istanbul University Central Campus, 34452 Beyazit, Fatih / Istanbul - Turkiye
Phone: +90 212 440 0000

EXPERIMED

INSTRUCTIONS TO AUTHORS

Context

Experimed is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official online-only publication of Istanbul University Aziz Sancar Institute of Experimental Medicine and it is published triannually on April, August, and December. The publication language of the journal is English.

Experimed aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of basic and clinical medical sciences. The journal publishes original articles, case reports, reviews, and letters to the editor that are prepared in accordance with ethical guidelines.

Editorial Policy

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Council of Medical Journal Editors (ICMJE), the World Association of Medical Editors (WAME), the Council of Science Editors (CSE), the Committee on Publication Ethics (COPE), the European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal conforms to the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be accepted for publication. Manuscripts submitted for evaluation should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been submitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization.

Peer-Review Policy

Only those manuscripts approved by its every individual author and that were not published before in or sent to another journal, are accepted for evaluation. Submitted manuscripts that pass preliminary control are scanned for plagiarism using iThenticate software. After plagiarism check, the eligible ones are evaluated by editor-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope. The selected manuscripts are sent to at least three national/international external referees for evaluation and publication decision is given by editor-in-chief upon modification by the authors in accordance with the referees' claims. Editor-in-chief evaluates manuscripts for their scientific content without regard to ethnic origin, gender, sexual orientation, citizenship, religious belief or political philosophy of the authors and ensures a fair double-blind peer review of the selected manuscripts.

Editor in chief does not allow any conflicts of interest between the authors, editors and reviewers and is responsible for final decision for publication of the manuscripts in the Journal.

Reviewers' judgments must be objective. Reviewers' comments on the following aspects are expected while conducting the review.

- Does the manuscript contain new and significant information?
- Does the abstract clearly and accurately describe the content of the manuscript?
- Is the problem significant and concisely stated?
- Are the methods described comprehensively?
- Are the interpretations and consclusions justified by the results?
- Is adequate references made to other Works in the field?
- Is the language acceptable?

Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the editor if they are aware of copyright infringement and plagiarism on the author's side.

A reviewer who feels unqualified to review the topic of a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

The editor informs the reviewers that the manuscripts are confidential information and that this is a privileged interaction. The reviewers and editorial board cannot discuss the manuscripts with other persons. The anonymity of the referees is important.

Ethical Principles

The Journal takes as principle to comply with the ethical standards of World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects and WMA Statement on Animal Use in Biomedical Research.

An approval of research protocols by the Ethics Committee in accordance with international standards mentioned above is required for experimental, clinical, and drug studies and for some case reports. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimental research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. It is the authors' responsibility to carefully protect the patients' anonymity. For photographs that may reveal the identity of the patients, signed releases of the patient or of their legal representative should be enclosed.

For studies involving animals, it is required to obtain approval of research protocols from an ethics committee. The committee reviews protocols to ensure compliance with applicable regulations and guidelines, including the Guide for the Care and Use of Laboratory Animals (8th Edition, 2011) and the International Guiding Principles for Biomedical Research Involving Animals (2012). These guidelines offer comprehensive instructions on how to carry out animal research ethically and humanely and are widely acknowledged as the benchmark for such research.

EXPERIMED

Authors should provide detailed explanation of the ethical treatment of animals in their manuscripts, including measures taken to avoid pain and distress. This is crucial to ensure the humane conduct of the study and enable verification that it conforms to the relevant ethical criteria. The ARRIVE checklist is a useful tool authors can use to present this information clearly and thoroughly.

Registration of Clinical Trials
Any research study (clinical trial) that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes' registration should be done following the guidelines at International Clinical Trials Registry Platform (ICTRP). The journal supports the development of registries following the related policy as explained at WAME's the Registration of Clinical Trials page.
The data of the studies that received ethics committee approval in Turkey are provided to the International Clinical Trials Registry Platform by the Ministry of Health. Information regarding ethics committee approvals are given in the articles of the journal.

Plagiarism
Experimed is extremely sensitive about plagiarism. All submissions are screened by a similarity detection software (iThenticate by CrossCheck) at any point during the peer-review or production process.Even if you are the author of the phrases or sentences, the text should not have unacceptable similarity with the previously published data.

When you are discussing others' (or your own) previous work, please make sure that you cite the material correctly in every instance.

In the event of alleged or suspected research misconduct, e.g., plagiarism, citation manipulation, and data falsification/fabrication, the Editorial Board will follow and act in accordance with COPE guidelines.

Authorship
Each individual listed as an author should fulfill the authorship criteria recommended by the International Committee of Medical Journal Editors

(ICMJE - www.icmje.org). The ICMJE recommends that authorship be based on the following 4 criteria:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

In addition to being accountable for the parts of the work he/she has done, an author should be able to identify which co-authors are responsible for specific other parts of the work. In addition, authors should have confidence in the integrity of the contributions of their co-authors.

All those designated as authors should meet all four criterias for authorship, and all who meet the four criteria should be identified as authors. Those who do not meet all four criterias should be acknowledged in the title page of the manuscript.
Experimed requires corresponding authors to submit a signed and scanned version of the authorship contribution form (available for download through http://experimed.istanbul.edu.tr/en/_) during the initial submission process in order to act appropriately on authorship rights and to prevent ghost or honorary authorship. If the editorial board suspects a case of "gift authorship," the submission will be rejected without further review. As part of the submission of the manuscript, the corresponding author should also send a short statement declaring that he/she accepts to undertake all the responsibility for authorship during the submission and review stages of the manuscript.

Conflict of Interest
The journal requires the authors and all individuals taking part in the evaluation process to disclose any existing or potential conflict of interest (such as financial ties, academic commitments, personal relationships, institutional affiliations) that could unduly influence one's responsibilities. To disclose potential conflicts of interest, the ICMJE Potential Conflict of Interest Disclosure Form should be filled in and submitted by authors as explained in the Author Form of the journal. Cases of a potential conflict of interest are resolved within the scope of COPE Conflict of Interest Flowcharts and ICMJE Conflict of Interest guidelines.

Besides conflict of interest, all financial support received to carry out research must be declared while submitting the paper.

The Editorial Board of the journal handles all appeal and complaint cases within the scope of COPE guidelines. In such cases, authors should get in direct contact with the editorial office regarding their appeals and complaints. When needed, an ombudsperson may be assigned to resolve cases that cannot be resolved internally. The Editor in Chief is the final authority in the decision-making process for all appeals and complaints.

Open Access Statement
The journal is an open access journal and all content is freely available without charge to the user or his/her institution. Except for commercial purposes, users are allowed to read, download, copy, print, search, or link to the full texts of the articles in this journal without asking prior permission from the publisher or the author. This is in accordance with the HYPERLINK "https://www.budapestopenaccessinitiative.org/read" BOAI definition of open access.

EXPERIMED

Disclaimer
Statements or opinions expressed in the manuscripts published in Experimed reflect the views of the author(s) and not the opinions of the editors, the editorial board, or the publisher; the editors, the editorial board, and the publisher disclaim any responsibility or liability for such materials. The final responsibility in regard to the published content rests with the authors.

MANUSCRIPT PREPARATION

The manuscripts should be prepared in accordance with ICMJE-Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (updated in December 2015 - http://www.icmje.org/icmje-recommendations.pdf). Authors are required to prepare manuscripts in accordance with the CONSORT guidelines for randomized research studies, STROBE guidelines for observational original research studies, STARD guidelines for studies on diagnostic accuracy, PRISMA guidelines for systematic reviews and meta-analysis, ARRIVE guidelines for experimental animal studies, and TREND guidelines for non-randomized public behavior.

Manuscripts can only be submitted through the journal's online manuscript submission and evaluation system, available at http://experimed.istanbul.edu.tr/en/_. Manuscripts submitted via any other medium will not be evaluated.

Manuscripts submitted to the journal will first go through a technical evaluation process where the editorial office staff will ensure that the manuscript has been prepared and submitted in accordance with the journal's guidelines. Submissions that do not conform to the journal's guidelines will be returned to the submitting author with technical correction requests.

Authors are required to submit the following:
- Copyright Agreement Form,
- ICMJE Potential Conflict of Interest Disclosure Form (should be filled in by all contributing authors)

during the initial submission. These forms are available for download at http://experimed.istanbul.edu.tr/en/_.

Preparation of the Manuscript
Title page: A separate title page should be submitted with all submissions and this page should include:
- The full title of the manuscript as well as a short title (running head) of no more than 50 characters,
- Name(s), affiliations, ORCID IDs and highest academic degree(s) of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number) and fax numbers, and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

Abstract: An English abstract should be submitted with all submissions except for Letters to the Editor. The abstract of Original Articles should be structured with subheadings (Objective, Material and Method, Results, and Conclusion). Please check Table 1 below for word count specifications.
Keywords: Each submission must be accompanied by a minimum of three to a maximum of six keywords for subject indexing at the end of the abstract. The keywords should be listed in full without abbreviations. The keywords should be selected from the National Library of Medicine, Medical Subject Headings database (https://www.nlm.nih.gov/mesh/MBrowser.html).

Manuscript Types
Original Articles: This is the most important type of article since it provides new information based on original research. The main text of original articles should be structured with Introduction, Material and Method, Results, and Discussion subheadings. Please check Table 1 for the limitations for Original Articles.

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses should be provided with a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be specified.

Units should be prepared in accordance with the International System of Units (SI).

Editorial Comments: Editorial comments aim to provide a brief critical commentary by reviewers with expertise or with high reputation in the topic of the research article published in the journal. Authors are selected and invited by the journal to provide such comments. Abstract, Keywords, and Tables, Figures, Images, and other media are not included.

Review Articles: Reviews prepared by authors who have extensive knowledge on a particular field and whose scientific background has been translated into a high volume of publications with a high citation potential are welcomed. These authors may even be invited by the journal. Reviews should describe, discuss, and evaluate the current level of knowledge of a topic in clinical practice and should guide future studies. The main text should contain Introduction, Clinical and Research Consequences, and Conclusion sections. Please check Table 1 for the limitations for Review Articles.

Case Reports: There is limited space for case reports in the journal and reports on rare cases or conditions that constitute challenges in diagnosis and treatment, those offering new therapies or revealing knowledge not included in the literature, and interesting and educative case reports are accepted for publication. The text should include Introduction, Case Presentation, Discussion, and Conclusion subheadings. Please check Table 1 for the limitations for Case Reports.

Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cas-

Table 1. Limitations for each manuscript type

Type of manuscript	Word limit	Abstract word limit	Reference limit	Table limit	Figure limit
Original Article	3500	200 (Structured)	30	6	7 or total of 15 images
Review Article	5000	200	50	6	10 or total of 20 images
Case Report	1000	200	15	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	5	No tables	No media

es, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

Tables

Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text.

Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

While citing publications, preference should be given to the latest, most up-to-date publications. Authors are responsible for the accuracy of references. References should be prepared according to Vancouver reference style. If an ahead-of-print publication is cited, the DOI number should be provided. Journal titles should be abbreviated in accordance with the journal abbreviations in Index Medicus/ MEDLINE/PubMed. When there are six or fewer authors, all authors should be listed. If there are seven or more authors, the first six authors should be listed followed by "et al." In the main text of the manuscript, references should be cited using Arabic numbers in parentheses. The reference styles for different types of publications are presented in the following examples.

Journal Article: Rankovic A, Rancic N, Jovanovic M, Ivanović M, Gajović O, Lazić Z, et al. Impact of imaging diagnostics on the budget – Are we spending too much? Vojnosanit Pregl 2013; 70: 709-11.

Book Section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR, editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a Single Author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

Editor(s) as Author: Huizing EH, de Groot JAM, editors. Functional reconstructive nasal surgery. Stuttgart-New York: Thieme; 2003.

Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

EXPERIMED

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Ö□rencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle □ndeksleri Kan Lipidleri Arasındaki Ili□kiler. H.Ü. Sa□lık Bilimleri Enstitüsü, Doktora Tezi. 2007.

Manuscripts Accepted for Publication, Not Published Yet: Slots J. The microflora of black stain on human primary teeth. Scand J Dent Res. 1974.

Epub Ahead of Print Articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. Diagn Interv Radiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlEID/cid.htm.

REVISIONS

When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

Editor in Chief: Prof. Dr. Bedia Cakmakoglu
Address: Istanbul University, Aziz Sancar Institute of Experimental Medicine, Vakıf Gureba Avenue, 34093, Capa, Fatih, Istanbul, Turkiye
Phone: +90 212 414 2000-33305
Fax: +90 212 532 4171
E-mail: bedia@istanbul.edu.tr

Publisher: Istanbul University Press
Address: Istanbul University Central Campus, 34452 Beyazit, Fatih / Istanbul - Turkiye
Phone: +90 212 440 0000