E-ISSN 2667-5846

# EXPERIMED

Volume **13** Issue **2 August** 2023

experimed.istanbul.edu.tr

ISTANBUL UNIVERSITY PRESS

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**INDEXING AND ABSTRACTING**

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**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

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# EXPERIMED

Experimed is proud to published the second issue of 2023 with fourteen valuable original research articles related to 20 different universities with several faculties and departments and multidisciplinary areas from Turkiye.

I am very appreciative of;

Researchers who have published their original scientific outcomes in Experimed,

Editorial Board Team who has conduct with great labour in all stages,

Dear Institute Director Prof. Dr. Günnur Deniz who has expressed her supportive gratitude for Experimed to University's management platform,

As a member of the Istanbul University Press team, Eda Kolukısa Doğru, Merve Ağırgün and Ertuğrul Yaşar worked hard to ensure timely publication.

We are very pleased that our growing influence in the literature with this amazing Experimed Team gives us more courage to shed more light on science with new publications.

Prof. Dr. Bedia Çakmakoğlu

Experimed 2023; 13(2): 73-79

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1264306

# Effect of Chronic Unpredictable Mild Stress on Hippocampus and Serum Markers in Rats

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**Cite this article as:** Yener MD, Colak T, Kurnaz Ozbek S, Ceylan S, Acar E, Kir HM. Effect of chronic unpredictable mild stress on hippocampus and serum markers in rats. Experimed 2023; 13(2): 73-79.

## ABSTRACT

**Objective:** Unpredictable stress is a common factor that we encounter in our daily lives. In this context, we wanted to investigate the morphological effects of a chronic unpredictable mild stress (CUMS) model on rat hippocampus and serum levels of brain derived neurotrophic factor (BDNF) and glial fibrillary acidic protein (GFAP).

**Materials and Methods:** This study was carried out on 16 Wistar albino rats which were divided into control and experiment groups. The CUMS model protocol was applied, and the morphological structures of the hippocampus were examined. The serum GFAP/BDNF levels were measured by ELISA method.

**Results:** The mean BDNF level was 1.65 ± 0.17 ng/mL in the control group and 2.25 ± 0.29 ng/mL in the CUMS group. The mean GFAP level was 3.04 ± 0.45 ng/mL in the control group and 2.96 ± 0.58 ng/mL in the CUMS group. When the polymorph cell layer (PCL) in gyrus dentatus was examined; the mean number of cells in the control group was 20 ± 1.38 for the left PCL and 17 ± 1.35 for the right PCL. In the stress group; the mean number of cells in the polymorph cell layer of gyrus dentatus was 27 ± 2.08 cells for the left side and 23 ± 2.01 for the right side.

**Conclusions:** As a result, morphological changes were observed in the gyrus dentatus region which has an important role in memory formation. The cells in the polymorph cell layer of gyrus dentatus underwent changes under stress.

**Keywords:** Chronic stress, hippocampus, brain derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP)

## INTRODUCTION

Homeostasis is the conservation of the organism's cellular balance against environmental factors (1). Stress is considered as a factor that disrupts homeostasis (2). In stressful situations, there are changes in the levels of many biomediators and at the same time, stress factors cause changes in various reflex communication pathways. These changes can cause morphological alterations in tissues that are highly sensitive to external factors such as the brain. The imbalance of these changes causes pathologies in living organisms (3).

The hippocampus area is related to the cognitive process of the brain such as memory and sense of direction in mammals (4). Gyrus dentatus is a morphological and functional neuroanatomic region of the hippocampus that consists of three basic morphological layers curled into each other in rats. These layers are molecular, pyramidal and polymorphic cell layers. Degeneration of the cells in these layers plays a part in the etiopathology of many neurological diseases (5).

Chronic stress conditions cause adaptations to regain homeostasis in the organism. These adaptations are biological steps that regulate the response to stress (6).

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**Submitted:** 13.03.2023 **Revision Requested:** 27.04.2023 **Last Revision Received:** 13.06.2023 **Accepted:** 14.06.2023 **Published Online:** 07.08.2023

Intermediary biological factors released from these pathways enable the rearrangement of many biological structures and adaptation of the physiological structure of the organism to the stress condition (2). If stress factors are at a level that disrupts cellular homeostasis and the adaptation process is insufficient, tissue and organ pathologies emerge. In particular, chronic stress may have irreversible consequences for the structural and functional integrity of the cell.

Neurons are in constant interaction with the microenvironment and are very sensitive to external stimuli. If the level of stress in which an organism interacts increases, disturbances in cellular biological pathways may occur (7). The adaptation mechanisms of the organism undergo reorganization to balance this situation (8). Neurons are surrounded by astrocytes and play an active role in many metabolic activities. Disturbances that may occur in astrocytes are directly related to neuron survival. Glial fibrillary acidic protein (GFAP) is an intermediary filament produced by the astrocyte cell throughout the central nervous system (CNS). In addition, an increased GFAP level is used as a marker for reactive astrogliosis pathology in animal models (9).

Neurotrophin is a fundamental signaling molecule in the CNS responsible for neuron growth, axon elongation, and the existence of a synapse during the developmental period. Also, the brain-derived neurotrophic factor (BDNF) protein is the most extensive growth factor in the CNS which is essential for neuronal survival (10). Along with CNS effects; BDNF, a neurotrophic peptide, also has peripheral effects and these effects are related to regulating the peripheral energy metabolism. This reasonably effective neurotrophic factor is being studied as a possible marker for the nervous system in mammals (11).

In this context, it was intended to investigate the morphological effects of chronic mild stress on hippocampal tissue and serum BDNF/GFAP levels in rats.

## MATERIALS AND METHODS

### Animals and Standard Procedures

In the study, four-months-old 190-200 gr female *Wistar albino* rats (n=16) were used. We divided the rats into two groups in standard cages two weeks before the initiation of the study process and provided standard room conditions. Menstrual cycles of rodents were considered and analyzed. During anesthesia; a combination of ketamine hydrochloride (90 mg/kg; Ketalar, Parke-Davis) and xylazine hydrochloride (12mg / kg, 2%; Rompun, Bayer) was administered intraperitoneally to the rodents. Euthanasia performed by cervical dislocation under anesthesia. Afterwards, intracardiac blood collection was performed. The principles of "Guidelines for the Care and Use of Laboratory Animals" were applied.

### Stress Model and Groups

The chronic unpredictable mild stress (CUMS) method was chosen as a stress protocol because it prevents rodents from learning stress factors and is a reliable method that has been used in many studies. In this study, the CUMS protocol which was previously defined by Willner P. (12) was used as a stress model. A total of eight different stressors were identified (Table 1) and the order of the stressors were previously determined. At the end of the experiment, anhedonia behaviors which are considered as an indicator of the depression status of the animals were closely monitored and measured.

**Table 1.** Stressors applied in CUMS model.

| | |
|---|---|
| 1. Cage inclination, 45°C / 24 hours | 5. Changing day-night cycle |
| 2. Hanging from the tail, 1 minute | 6. Cage shaking, 10 minutes |
| 3. Buoyancy in cold water 4°C / 5 minutes | 7. Cage wetting 200 mL / 24 hours |
| 3. Buoyancy in hot water 45°C / 5 minutes | 8. Exchanging sawdust between cages |

In this study, two groups were formed as the experimental group and control group. Then we applied the stressors to the experimental group for 28 days. The order of the application protocol was randomly determined before the start of the experiments.

### Sucrose Consumption Test

During the 4th week of the experiment, a sucrose preference test was conducted to measure anhedonic behaviors in stressed animals. For the test, two water bottles were placed in each cage and applied for six days. One bottle contained 200mL of 20% sucrose solution, and the other contained 200mL of tap water. The rats were allowed to drink from both bottles during the first five days for habituation. The water bottles were changed every 12 hours. Sucrose consumption was calculated as the ratio of sucrose consumption to total consumption: Sucrose consumption = (sucrose consumption x 100) / total consumption.

### Histological Preparation and Procedure

Brain tissues were fixed in 10% buffered formaldehyde for a week. Histological preparations were performed as previously described (13). Brain tissues were dehydrated through upgrading levels of ethanol solutions and cleared in xylene then embedded in paraffin. Before proceeding to the histological serial sectioning stage, the coordinates of the desired cross-sectional area were determined according to the region of bregma (Bregma -3.00 mm) using the brain atlas (*Paxinos, Watson*) of the hippocampus region of the animals whose brain tissues were completely removed. Sections of the brain tissues were stained with hematoxylin and eosin to determine the hippocampus microstructure. The end boundary of the pyramidal cell layer in the CA3 region within

the hippocampus tissue was determined as the counting area and a border perpendicular to the tissue axis was drawn at this point. Cell count was performed in the region between this axis and the granular cell layer of the hippocampus.

## ELISA Method and Biochemical Procedures

Blood samples (3mL) were obtained from the left ventricle. Blood specimens were allowed to clot for two hours at room temperature and centrifuged for 15 minutes at 1000 g force at 4-8$^0$C. The supernatants were collected and diluted 1/10 before the assay. Serum samples were stored at -40°C. The serum BDNF and GFAP levels were determined with Enzyme Linked Immunosorbent Assay (ELISA; Elabscience USA) kits were used and measured with the Alisei Quality System Seac Radim Company analyzer (Italy/Rome)-ELISA reader on the basis of the manufacturer's instructions (BDNF, Catalog number E-ELR1235; GFAP, Catalog number E-ELR1428). The results were multiplied by the dilution coefficient and their concentrations were calculated according to the kit standards.

## Data Analysis and Statistics

A Kolmogorov-Smirnov analysis was performed for the normal distribution suitability test in the statistical evaluation of the study results. An independent t-test was applied for the values that corresponded to normal distribution. The Mann Whitney U test was used for the parameters that did not comply with normal distribution. The p values of 0.05 or less were considered statistically significant. The statistical analysis SPSS 22.0 (IBM, Packet program) was used. The GraphPad Prism8 package program was used for graphics design.

## RESULTS

During the study, animal weights were monitored, and the mean weights at the beginning of the study were 193 gr for the control group and 198 gr for the stress group. Body mass increase was less in rats under chronic stress protocol when compared to the control group. Even so there was no significant difference between the mean weights of the groups. The temporal change graph of the mean values of animal weights according to the groups is given in Figure 1.

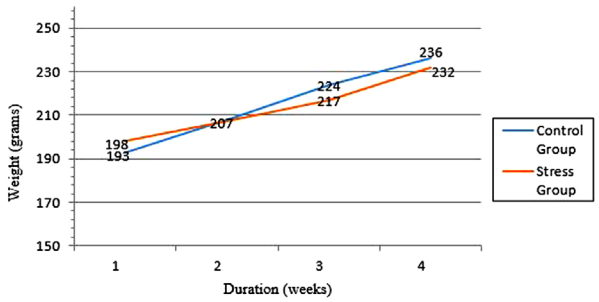


**Figure 1.** Variations in the average weight of animals according to the groups and time.

The sucrose consumption test, which is an important parameter in stress model validation, was applied to the rodents. In the study, there was a significant decrease in sucrose consumption when the rats underwent the CUMS procedure as compared to the control group ($p< 0.05$).

We performed a histological evaluation of the hippocampus tissue separately for the right and left sides. We measured the hippocampus thickness for both groups on the vertical axis 'coronal section' and compared them statistically. The molecular, pyramidal and polymorphic area thicknesses with complete hippocampus tissue thicknesses and subdomains were also evaluated among the groups. The mean thickness of the left hippocampus was 1,581 ± 61.01 µm for the control group. In the stress group rats, the mean left hippocampus thickness was 1,486 ± 48.94 µm. The mean thickness of the right hippocampus was 1,544 ± 36.75 µm for the control group. In the stress group rats, the mean right hippocampus thickness was 1,478 ± 91.82 µm. Figure 2 shows the right and left hippocampal thicknesses according to the groups.

When the hippocampus thicknesses of the rats in the experimental and control groups were compared statistically, no significant difference was found in terms of their thickness ($p>0.05$). Between the groups; no significant difference was found between molecular, pyramidal and polymorphic area thicknesses ($p<0.05$). When the polymorphic cell layer (PCL) in the gyrus dentatus was examined, the average number of cells was 20 ± 1.38 cells/field for the left PCL and 17 ± 1.35 cells/field for the right PCL in the control group. In the stress group, the average number of cells in the polymorphic cell layer of the gyrus dentatus was 27 ± 2.08 cells/field for the left side and 23 ± 2.01 cells/field for the right side. The cell count in the polymorph cell layer of gyrus dentatus of the hippocampus is given in Figure 3.

When the gyrus dentatus regions were examined, there was a statistically significant difference among the groups cell count in the PCL ($p<0.05$). When the stress group and control group were compared for the left hippocampus, there was a significant increase in the cell count in the relevant area of the stress group compared to the control ($p=0.021$). A statistically significant increase in the cell count in the dentate gyrus of the rodent brain in the stress group was found as compared to the control for the right hippocampus ($p=0.026$). When both experimental groups were compared statistically, there were no significant differences between cell counts in the gyrus dentatus regions of the right and left hemispheres ($p>0.05$).

The obtained histological images were examined morphologically in both groups. It was observed that the cells in the polymorph cell layer of the animals belonging to the stress group were morphologically spindled. Furthermore, there was a statistically significant increase in the polymorph cell count in the stress group ($p<0.05$). In the PCLs of the rodents belonging to the stress group, the cells increased with a spindle morphological structure. The histological images of the stress and control groups are shown in Figure 4.

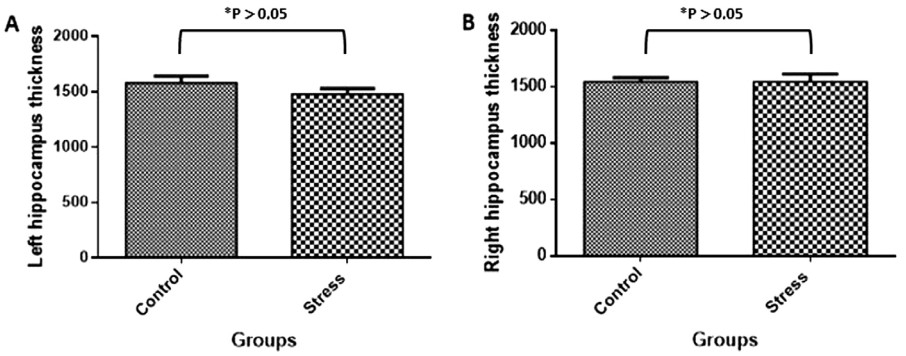


**Figure 2.** Right and left hippocampus thicknesses for groups. A. Inter-group hippocampal thickness for left hippocampus coronal section, B. Inter-group hippocampal thickness for right hippocampus coronal section.

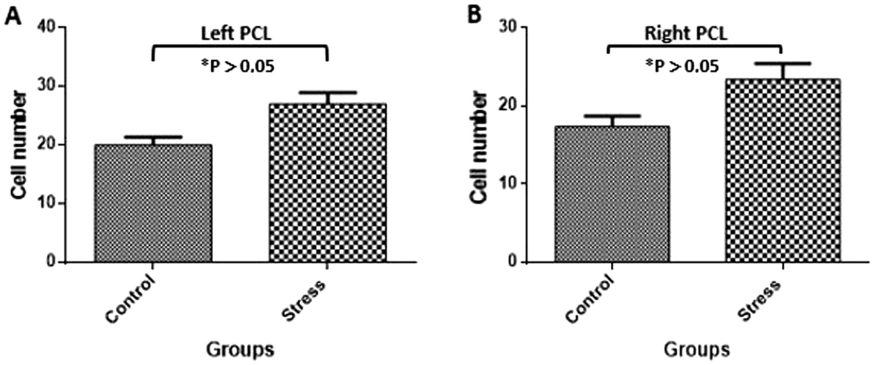


**Figure 3.** Cell count in the polymorph cell layer, gyrus dentatus. A. Inter-group cell count for the left PCL in gyrus dentatus, B. Inter-group cell count for the right PCL in gyrus dentatus.

We performed the BDNF and GFAP measurements via the ELISA method in serum obtained after intracardiac blood collection. The mean BDNF level was 1.65 ng/mL in the control group and 2.25 ng/mL in the CUMS group. The mean GFAP level was 3.04 ng/mL in the control group and 2.96 ng/mL in the CUMS group. There was no statistically significant difference among the groups in terms of the BDNF and GFAP serum levels ($p>0.05$, Figure 5).

## DISCUSSION

Stress has a significant impact on the brain, and it can cause changes in brain function and structure. The response of stress hormones affects various brain regions, particularly the hippocampus, which is crucial for memory formation. The hypothalamic-pituitary-adrenal (HPA) axis is the primary biological pathway for regulating the effects of stress on the organism. Stress hormones which a steroid structure secreted through this system makes radical changes in the metabolism (3). A thorough understanding of the mechanism of the action of stress can be an effective way to treat diseases such as neuron degeneration.

It is known that stress affects neurobiological pathways and in this way affects neurons, astrocytes and microglias (14). During this process; neurons, astrocytes and glial cells are affected by the HPA axis by means of immunological pathways, and neurotrophic factors. One of the most characterized neurotrophic factors is BDNF, which modulates brain health and neuronal survival (11). The activity dependent expression of BDNF is a crucial mechanism for synaptic plasticity that improves a mammals' learning ability (15). This study investigated whether there was a correlation between BDNF, which has important effects on the biological pathways of CNS and the morphology of the hippocampus under stress. In this study, no difference was found between the experimental group of rats BDNF serum levels which had been applied with the CUMS. The GFAP is a structural filament protein that is mainly expressed in astrocytes (9). The astrocytic cytoskeleton filament protein GFAP plays a role in many processes in the CNS. It has important effects, especially on neuronal survival. Astrocytes have critical roles in maintaining neuronal metabolism, regulating neuroimmunological reactions and maintaining the blood-brain barrier. GFAP, an astrocyte marker,

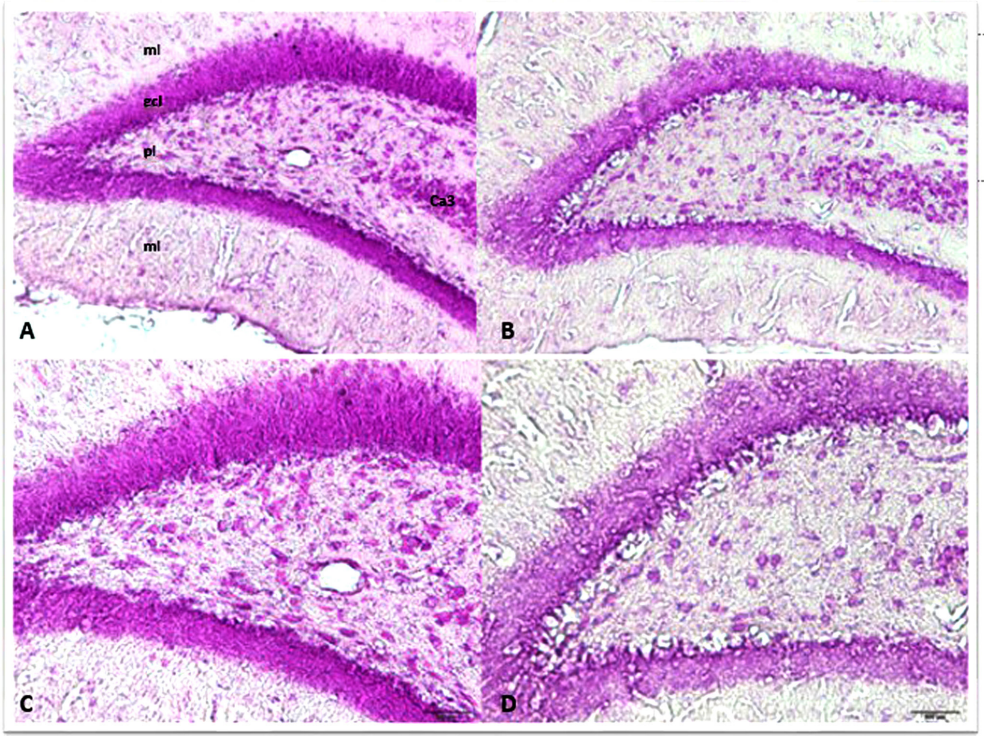


**Figure 4.** Microscopic images of gyrus dentatus and subunits of CUMS and control group. A. CUMS group gyrus dentatus, 200X; B. Control group gyrus dentatus, 200X; C. CUMS group gyrus dentatus, 400X; D. Control group gyrus dentatus, 400X; pl: Polymorph layer; gcl: Granular cell layer; ml: Molecular layer; Ca3: Cornu ammonis3.

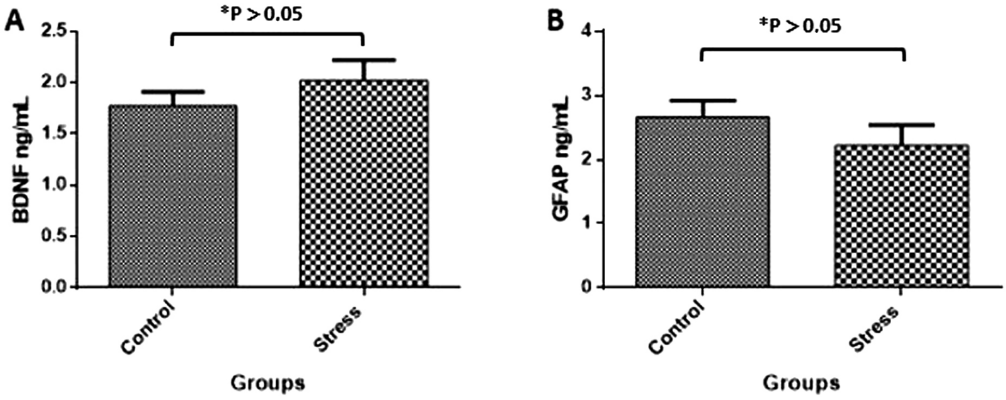


**Figure 5.** Serum BDNF and GFAP levels for CUMS and control groups. A. Intergroup comparisons of serum BDNF levels, B. Intergroup comparisons of serum GFAP levels.

increases expression in a brain injury or CNS degenerations (16). In this context; we examined the GFAP serum levels as a biomarker for astrocytes, an important component of CNS, as well as hippocampus morphology. There were no significant differences in the GFAP serum levels among the groups.

Excessive stress is a phenomenon that damages the brain tissue. The hippocampus is damaged both cognitively and morphologically under this condition (6). It is even reported that there is a decrease in hippocampal volume with chronic depression. This volumetric reduction was reported to be due to the pyramidal cell depletion in the cornu ammonis region (17). The organism activates an adaptation mechanism in response to stress. However, the hippocampus also degenerates during chronic stress conditions that disrupt hemostasis (10). The most devastating outcomes occur during chronic severe

stress situations where the adaptation mechanism of the cell is inadequate. Cellular degeneration is inevitable in prolonged periods of intense stress (18). However, it should be noted that mild stress provides cellular stimulation and keeps the organism alert (19). It is reported that neurogenesis decreases during chronic severe stress conditions and increases in acute stress conditions in the hippocampus area. *In vivo* studies showed that the hippocampus also increased neurogenesis in single dose corticosterone administration. Other studies showed that stress hormones cause an increase in fibroblast growth factor (FGF2) receptors in rats hippocampal cells (20). In this study, no difference was found between the hippocampus thickness of rats. The biological pathways underlying the different response mechanisms of animals to stress is thought to contribute to the development of treatment options for stress-dependent diseases. In this context; we applied a mild stress model that is frequently faced in daily life and used an unpredictable experimental model. We prevented the animals from getting accustomed to stress and adapting to it. We examined the morphological changes and cell count changes in the polymorph cell layer in the hilum of the dentate gyrus area under chronic unpredictable mild stress. This study observed a cellular increase in morphologically spindled cells in the region of the PCL. We determined that there was a statistically significant increase in the cell count in the PCL. The close relationship between changes in this area and memory is emphasized in the literature. Degenerations in this part of the gyrus dentatus are closely associated with memory disorders (21).

In another *in vivo* study; when the hippocampal neurogenesis of rodents was inhibited under stress conditions, temporal prolongation in the decrease of stress hormones to normal serum levels was observed. In other words, inhibited neurogenesis was associated with elevated stress-induced high glucocorticoid levels for longer periods. Hippocampal neurogenesis was suggested to buffer the stress responses and depressive behaviors in mammalians (22). It is suggested that hippocampal neurogenesis has a buffer function against stress conditions, in mice (19). In this study, we determined cellular changes between the groups 'stress/control' in the PCL of gyrus dentatus against mild stress. The cellular increase in the stress group is considered to be part of an adaptation mechanism to stress, which is also mentioned in the literature. Possible cellular increases in the polymorph cell layer under chronic unpredictable stress may bring about new cellular connections. In this case, it is thought that new cells originating from the progenitor area may cause memory disorders. Newly formed cells can create new synapses independent of the information flow and create new pathways in memory formation in this area. This process is formed as a result of the organism's struggle with stress and can create disruptions in memory condensation (21). This could provide a neurobiological approach to explaining post-stress memory disorders and contribute to improving treatment options.

## CONCLUSION

This study aimed to determine how CUMS morphologically affects the hippocampus of rodents. In addition, we evaluated the serum values of BDNF and GFAP which are accepted as biological markers for neurons and astrocytes with morphological changes. When we compared the stress and control groups, we observed changes in the hippocampus of animals under stress conditions. These changes were observed in the gyrus dentatus region which has an important role for memory formation. The cells in the PCL of gyrus dentatus underwent changes under stress. In the results obtained, there was an increase in the cell count in the PCL of gyrus dentatus in the stress group. In addition, the cellular morphologies changed and became spindle shaped. However, the study did not find any significant difference when CUMS and the control groups were compared in terms of hippocampus thickness. In addition, there was no significant difference in the BDNF and GFAP serum markers levels between the groups.

**Ethics Committee Approval:** Ethical approval of the study was obtained from Animal Experiments Local Ethics Committee, Kocaeli University (2015/44).

**Authors' Contributions:** Conception/Design of Study - M.D.Y., T.C.; Data Acquisition – M.D.Y., S.K.O., E.A.; Performing experiments – M.D.Y., S.K.O., E.A.; Data Analysis/Interpretation – M.D.Y, T.C.; Statistical Analyses – M.D.Y., S.K.O., E.A.; Drafting Manuscript – M.D.Y., T.C.; Critical Revision of Manuscript – M.D.Y., T.C., S.K.O., S.C., E.A., H.M.K.; Final Approval and Accountability - M.D.Y., T.C.

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

**Financial Disclosure:** This study supported by Scientific Research Projects Coordination Unit, Kocaeli University (2016/032).

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Experimed 2023; 13(2): 80-85

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1268503

# Gingipain Injection Affects Intestinal Oxidant-Antioxidant Status and Alkaline Phosphatase in Overfed Zebrafish

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**Cite this article as:** Gunduz G, Beler M, Unal I, Cansiz D, Emekli-Alturfan E, Kose KN. Gingipain injection affects intestinal oxidant-antioxidant status and alkaline phosphatase in overfed zebrafish. Experimed 2023; 13(2): 80-85.

## ABSTRACT

**Objective:** *Porphyromonas gingivalis* (*P. gingivalis*), a major periodontopathogen, is associated with overfeeding disorders, including metabolic syndrome. Gingipains are one of the most powerful endotoxins of *P. gingivalis*. Our aim was to reveal the effects of gingipain injections on the intestinal oxidant-antioxidant status and alkaline phosphatase (ALP) activity in overfed zebrafish.

**Materials and Methods:** Four groups of healthy adult zebrafish were placed in random tanks as C: Control (n=15); GP: Gingipain (n=15); OF: Overfeeding (n=15); and OF+GP: Overfeeding+Gingipain (n=15) groups. At the end of the experiment, levels of intestinal lipid peroxidation (LPO) and ALP, glutathione S-transferase (GST), and catalase (CAT) activities were evaluated.

**Results:** Intestinal LPO was significantly lower in the GP and OF groups compared to C. Gingipain injection in OF (OF+GP) significantly elevated LPO when compared to C, GP, and OF groups. ALP activities decreased significantly in the GP, OF, and OF+GP compared to C. GST activities increased significantly in the GP when compared to C. Decreased GST activities were observed in the OF and OF+GP. This decrease was less in OF+GP. CAT activities significantly decreased in all groups when compared to C.

**Conclusion:** Our findings demonstrate that gingipain injection alters the ALP activity and intestinal oxidant-antioxidant status in overfed zebrafish.

**Keywords:** Gingipain, zebrafish, oxidative stress

## INTRODUCTION

*Porphyromonas gingivalis (P. gingivalis),* a gram-negative anaerobic bacteria, is involved in the etiology of periodontitis. Periodontitis is generally characterized by bacteria-induced inflammation, which damages the tissues around teeth resulting in teeth loss. This strong keystone periodontopathogen, highly related to severe periodontitis, is an effective colonizer of the oral epithelium. *P. gingivalis* can infiltrate periodontal tissues and get over the host's immune response. Using a panel of virulence factors, it destabilizes innate immunological and inflammatory responses (1). Capsule, lipopolysaccharide (LPS), fimbriae, and cysteine proteases are some listed virulence factors of *P. gingivalis* (2).

*P. gingivalis'* extracellular cysteine proteinases, known as gingipains, are major endotoxins among these virulence

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**Submitted:** 24.03.2023 **Revision Requested:** 24.04.2023 **Last Revision Received:** 02.05.2023 **Accepted:** 05.05.2023 **Published Online:** 03.08.2023

Experimed 2023; 13(2): 80-85

factors. The gingipains are composed of Lys-gingipain (Kgp) and Arg-gingipain (Rgp) (RgpA and RgpB). Gingipain R is specific for arginine, while gingipain K is specific for lysine residues. Gingipains cleave amino acid chains right after lysine or arginine residues at the C-terminal region. In the physiology of the bacteria, gingipains' proteolytic activity is critical for adhering to host surfaces, obtaining nutrition through protein degradation, and promoting further colonization. While present in circulation, gingipains are also associated with fibrin(ogen)olytic activity and can interact with and degrade plasma proteins (3). Gingipains are responsible for 85% of *P. gingivalis'* extracellular proteolytic activities and play a major role in periodontitis. They activate host matrix metalloproteinases, inactivate immune defense by degrading immunological components, and cleave immune cell receptors, causing immune dysregulation and inflammation (2).

*P. gingivalis* and its endotoxins, including gingipains are detected in circulation and organ lesions (4, 5). The host defense molecules produced against these endotoxins together with bacteria itself play a significant role in the relationship between systemic diseases and periodontitis (6). The current trend topic for periodontal research is the connection between periodontitis and metabolic syndromes, including obesity and diabetes mellitus (7, 8). Recent research has also suggested that *P. gingivalis* cause an imbalance in the microbiota of intestinal tissues and exacerbate metabolic problems (9).

The metabolic syndrome, formerly referred to as insulin resistance, is a group of risk factors including elevated triglycerides and fasting glucose, reduced high-density lipoprotein, high blood pressure and abdominal obesity that increase the risk of type 2 diabetes mellitus (T2D), cardiovascular diseases and even death. In metabolic syndrome, inflammation in adipose tissues and imbalance in intestinal flora are thought to be possible reasons in this abnormal systemic status. As a consequence of continuous exposure to high-fat diets, the composition of gut microbiota is altered and the intestinal mucosa barrier is damaged, allowing enterotoxins to enter systemic circulation. Rapid adipocyte hypertrophy caused tissue hypoxia and induced elevated adipokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), stimulating macrophages to release pro-inflammatory substances resulting in increased systemic inflammation and insulin resistance (9).

Intestinal alkaline phosphatase (ALP) is a major player in maintaining intestinal homeostasis and health. Obesity is one of the chronic inflammatory conditions that have been linked to altered ALP expression. Intestinal ALP is essential in the activation of bacterial Lipopolysaccharides (LPS) through dephosphorylation of its lipid A moiety, resulting in a non-toxic monophosphoryl section. This dephosphorylated monophosphoryl lipid A is not capable of forming a complex with the host's toll like receptor 4 (TLR4) (10).

Oxidative stress, the imbalance between oxidants and anti-oxidants, as a result of increased free radicals is involved in the molecular mechanisms underlying many diseases. In order to countervail these harmful molecules, the anti-oxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione peroxidase in addition to antioxidant molecule, glutathione (GSH) are produced.

The increased oxidative stress is considered to be an essential factor in the relation between periodontitis and over-eating induced-metabolic syndrome. Intestinal homeostasis and health are also involved in the molecular mechanisms related with obesity and metabolic syndrome. Increased serum Reactive oxygen species (ROS) are present in these conditions, and a pro-inflammatory state is expected to have a reciprocal influence on the molecular mechanisms (7). Patients with periodontitis may experience greater levels of oxidative stress induced-inflammation due to the deranged endogenous antioxidant defense system driven by the overproduction of lipid peroxidation products at inflammatory sites (11).

Due to their various benefits, including their small size, brief life cycle, availability in huge numbers, and low maintenance requirements, zebrafish have become a prominent vertebrate model organism for biomedical research. Zebrafish are used in numerous areas, including genetics, biomedicine, neuroscience, toxicology, pharmacology, as well as the modeling of human disease (12). Overfeeding-caused metabolic syndrome diseases such as obesity and diabetes have been well documented in zebrafish models as well (13-15). *P. gingivalis* and gingipains related research have been recently conducted in zebrafish, but in these studies and other animal models, the specific effects of gingipains have not yet been explored. Despite *P. gingivalis'* many other virulence factors such as LPS, capsule, and fimbria, which all create a cumulative effect in bacterias' pathogenicity, it is important to underline the specific effects of gingipain itself in order to reveal this powerful endotoxins' toxicity (16-18). Here in this study, our aim was to assess the impact of gingipain injections on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish to explore the relationship between periodontopathogens' virulence factors and metabolic diseases and to underline the role of gingipain in these complicated pathological mechanisms. We also assessed intestinal ALP because of its regulatory role in intestinal homeostasis in the case of overfeeding situations, which is an inflammatory condition related with altered ALP expression.

## MATERIALS AND METHODS

### Animals and Treatment

The animal experiments conducted in this study were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). The entire procedures used were authorized by the Marmara University Animal Care and Use Committee (38.2021mar). According to the ARRIVE (Animal Research: Reporting of *in Vivo* Experiments)

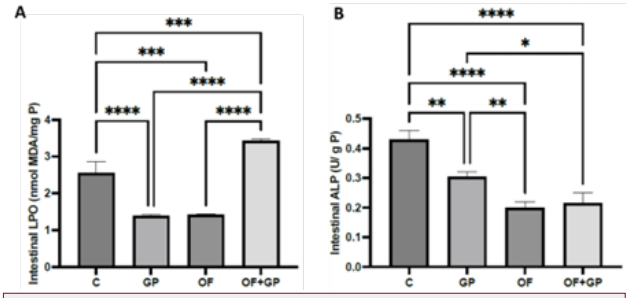


**Figure 1. A:** LPO levels **B:** ALP activities of the groups. Values are given as means ± SD; n= 6. **** p<0.0001; *** p<0.001, ** p<0.01; * p<0.05. LPO: Lipid peroxidation, ALP: Alkaline phosphatase, C: Control; GP: Gingipain, OF: Overfeeding, OF+GP: Overfeeding+Gingipain,

guidelines, care was taken to utilize as few animals as possible in the research. Zebrafish (AB/AB strain, wild type) were kept in an aquarium rack system (ZebTEC, Tecniplast, Italy) at a 14/10-hour light/dark cycle and 27–28 ± 1$^0$C temperature. Four groups of zebrafish (4-6 months old) were placed at random as C: Control (n=15); GP: Gingipain (n=15); OF: Overfeeding (n=15); and OF+GP: Overfeeding+Gingipain (n=15) groups. The control group was fed with 20 mg of fish food/fish/day, while the overfed zebrafish groups were fed with 120 mg/fish/day of commercial fish food (3.39 kcal/g; TetraMine; Tetra; Germany) over six daily feeding periods using an automated feeding system (Yinsheng T8800; Yinsheng; China). Overfeeding procedure was continued for 15 days. The content of tetramine consisted of a minimum of 51% crude protein, 11% crude oil, 2.3% calcium, 1.5% phosphorus and a maximum of 3% crude fiber, 6.5% moisture and 15% ash. The granule proportions of tetramine were between 0.65 mm and 0.36 mm. The tanks were cleaned and filled with fresh water daily. Gingipain (MyBioSource, Inc., USA, MBS969681, Recombinant *P. gingivalis* Gingipain R1(rgpA), partial) at 186 nmol/L was intraperitoneally injected into fish in the GP group. Gingipain dosages were chosen and applied in accordance with range finding research (19). 6 hours after injection applications the zebrafish were sedated and euthanized. Intestinal tissues of the zebrafish were taken, and for upcoming analyses, replicate samples of the tissues were made.

## Biochemical Analyses

The total protein concentrations were measured using the Lowry et al. method, and the findings were provided per protein (20). Malondialdehyde (MDA) levels were measured using the Yagi method, and they were analyzed as thiobarbituric acid reactive compounds that are formed as byproducts of lipid peroxidation (LPO) (21). CAT activity was assessed by using the modified method of Aebi (22). For the conjugation of GSH, GST is required, and the activity of GST was measured using a spectrophotometer at 340 nm (23). ALP activity was measured according to Walter and Schutt method (24).

## Statistical Analyses

For the statistical analyses GraphPad Prism 9.0 (GraphPad Software, San Diego, USA) was used and the data were presented as mean ± standard deviation. Dunn's multiple comparison test was run after the Kruskal Wallis test to compare the data. The Pearson correlation test was applied to evaluate the relationship between the oxidant and antioxidant parameters. Significant data were defined as those with a p-value lower than 0.05 (p<0.05).

## RESULTS

When compared with the control group, overfeeding and gingipain injections yielded a significant decrease in intestinal LPO (p<0.001 and p<0.0001, respectively). However, gingipain injections increased LPO level significantly in overfed zebrafish (OF+GP) when compared to the GP, OF, and control groups, (p<0.0001, p<0.001, and p<0.001, respectively) (Figure 1A).

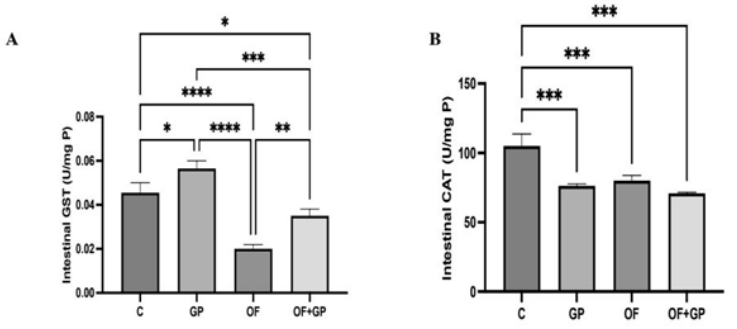


**Figure 2. A:** GST **B:** CAT activities of the groups. Values are given as means ± SD; n= 6. **** p<0.0001; *** p<0.001, ** p<0.01; * p<0.05. GST: Glutathione S-transferase**,** CAT: Catalase, C: Control; GP: Gingipain, OF: Overfeeding, OF+GP: Overfeeding+Gingipain

Intestinal ALP activities decreased significantly by gingipain injections and overfeeding in all three groups (GP, OF, and OF+GP) when compared to the control group ($p<0.01$, $p<0.0001$, and $p<0.0001$, respectively) (Figure 1B).

Intestinal GST activities were significantly elevated by gingipain injections when compared with the control group ($p<0.05$). However, decreased GST activities were observed in the OF and OF+GP groups ($p<0.0001$ and $p<0.05$, respectively). Gingipain injections led to increased GST activities in the overfed zebrafish (OF+GP) ($p<0.01$) (Figure 2A).

Intestinal CAT activities decreased significantly by gingipain injections and overfeeding in all three groups (GP, OF, and OF+GP) when compared with the control group ($p<0.001$). No significant change was observed among GP, OF, and OF+GP (Figure 2B).

In the GP group, LPO was positively correlated with CAT activity ($r=1$; $p<0.0001$), and negatively correlated with GST activity ($r=-1$; $p<0.0001$). In the OF group, LPO was positively correlated with GST activity ($r=1$; $p<0.0001$); whereas a negative correlation was found between LPO and CAT activity in the same group ($r=-1$; $p<0.0001$). In the OF+GP group, LPO was negatively correlated with CAT and GST activities ($r=-1$; $p<0.0001$).

## DISCUSSION

*P. gingivalis* and one of its most significant endotoxins, gingipain, have been detected in organs and the circulatory system. The inhibition of gingipains with the specific inhibitors in *P. gingivalis* infected mice reduced neuroinflammation and brain infection (4). Oral administration of *P. gingivalis* exacerbates the severity of drug-induced colitis in mice (5). These and many other data demonstrate that *P. gingivalis*, key periodontopathogen, and its endotoxins are associated with many systemic disease pathologies. The current trend topic for periodontal research is the relation between periodontitis and metabolic syndromes, including obesity and diabetes mellitus, which are related with overfeeding (7, 8). Many studies about the relation with periodontopathic bacteria and intestinal disorders have indicated that *P. gingivalis* generate the dysbiosis of gut microbiota resulting in the aggregation of metabolic disorders, recently (9).

Dynamic relationships between the intestinal epithelium, the microbiota, and the human immune system are necessary for intestinal homeostasis. Intestinal homeostasis is maintained by a variety of regulatory mechanisms, and a malfunction in these pathways may lead to the chronic inflammatory pathological evident in inflammatory bowel disease (25).

The oral administration of *P. gingivalis* dramatically worsened colitis in the experimental colitis model. By lowering the expression of tight junction proteins *in vivo*, the ingested *P. gingivalis* damaged the colonic epithelial barrier in mice. The disruption of the epithelial barrier specific to *P. gingivalis* was shown by *in vitro* permeability experiments employing the intestinal epithelial cell line (26).

In this study, we wanted to assess the impacts of gingipain injection on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish. We found that gingipain injections decreased intestinal ALP activity. Through interactions with the local microbiota, nutrition and the gut, intestinal ALP has an important function in intestinal homeostasis and health. The function of intestinal ALP in the intestine is to dephosphorylate extracellular nucleotides like uridine diphosphate as well as harmful microbial ligands such as LPS. Intestinal ALPs' capacity to control the microbial environment by establishing a complicated interaction between the microbiota, food, and the intestinal mucosal surface is also crucial in these issues. The gut microbiota and homeostasis can be affected by overfeeding because it changes intestinal ALP expression and activity (27).

Obesity is one of the chronic inflammatory conditions that have been linked to altered intestinal ALP expression (10). The results of our study showed decreased intestinal ALP activity in the overfed group. This finding indicates the diminished protective effect of intestinal ALP due to overfeeding on intestinal hemostasis.

In this study, the activities of CAT and GST were evaluated as they are the main antioxidant enzymes that might detoxify free radicals in the case of GP injection and overfeeding. CAT enzymes defend cells against free radical damage by degrading hydrogen peroxide (22), and GSTs may detoxify the end products of LPO and they can prevent the formation of lipid hydroperoxides (23).

Gingipain injections decreased intestinal CAT activity and LPO in the intestines. The reduction in CAT activity may be associated with the use of CAT to inhibit the increase in LPO associated with gingipain toxicity-induced inflammation. Similarly, the decreased LPO levels in the OF group may be associated with decreased CAT activity in the same group due to its use as an antioxidant to prevent the increase in LPO, consistent with the negative correlation between LPO and CAT observed in this group. Decreased LPO in the GP injected group may also be attributed to the substantial increase in the GST activities in the same group. In order to combat foreign compounds or oxidative stress products, GSTs catalyze the conjugation of reduced glutathione, resulting in the formation of less reactive molecules that are easily eliminated (28). Accordingly, in our study, increased GST in the gingipain-injected group may be suggested to prevent the increase in LPO levels due to gingipain injection. Decreased GST activity in the overfed group is consistent with the reported decrease in the adipose tissue GST enzyme content of high fat fed mice (29). However, gingipain injections in the overfed group caused an elevation in the GST activities to overcome oxidative stress induced by gingipain, as evidenced by increased LPO in the same group. Moreover, gingipain injections caused a significant elevation in GST activity, whereas overfeeding caused a reduction in

intestinal GST activity. Lower GST activity in the gingipain-injected overfed group, when compared with the gingipain-injected group, demonstrated that overfeeding diminished antioxidant response and even the gingipain challenge was not enough to augment this impairment.

As far as we are aware, there are only a few studies that have used gingipain *in vivo* models directly (18). It would be advantageous to increase the number of studies on the direct use of gingipain in zebrafish, given the benefits of this animal model, including their use in toxicological studies and the modelling of systemic diseases. Moreover, to our knowledge, this is the first study to report the effects of gingipain injections on the intestinal oxidant-antioxidant status and ALP activity in overfed zebrafish. Our study showed that gingipain disrupts the oxidant-antioxidant balance and ALP activity in the intestines due to overnutrition and provided important information on the effects of periodontal pathogens on intestinal health in metabolic diseases. We believe that our findings will guide future studies related to the connection of systemic diseases and periodontitis pathology.

**Ethics Committee Approval:** This study is approved by of Marmara University Animal Experiments Local Ethics Committee (38.2021mar).

**Authors' Contributions:** Conception/Design of Study- G.G., E.E.A., K.N.K.; Data Acquisition – G.G., M.B.; Data Analysis/Interpretation – G.G., M.B., İ.Ü., D.C., E.E.A., K.N.K.; Drafting Manuscript– G.G.; Critical Revision of Manuscript- E.E.A., K.N.K.; Final Approval and Accountability– G.G., M.B., İ.Ü., D.C., E.E.A., K.N.K.

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

**Financial Disclosure**: This project was supported by Marmara University Scientific Research and Project Commission, Project No: TDK-2021- 10420.

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Experimed 2023; 13(2): 86-92

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1260973

# Evaluation of DNA Microarray in Biomarker Detection in Cell-free DNA from Colorectal Cancer Cell Lines: A Proof-of-Concept Study

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**Cite this article as:** Kazan HH, Karahan CP, Celik E, Ozketen AC, Birgucu Cagil D, Ergun MA. Evaluation of DNA microarray in biomarker detection in cell-free DNA from colorectal cancer cell lines: A proof-of-concept study. Experimed 2023; 13(2): 86-92.

## ABSTRACT

**Objective:** DNA microarray is a powerful method to identify genomic anomalies including small insertions, duplications and/or deletions. This method is widely used in routine genetic screening for explaining the genetic background of certain phenotypes, for example, cancer. Cell-free DNA (cfDNA), which is an approach that may give information about the somatic tissues in peripheral blood, is another popular method used in routine genetic screening to understand the background of particular phenotypes, one of which is cancer. There is limited available research that investigates the involvement of these two approaches to decipher novel cancer biomarkers in the literature. However, detection of cancer biomarkers, especially non-invasive types, has been of great interest to research groups.

**Materials and Methods:** In the present study, we used colorectal cancer as a model tumor to figure out whether we could determine definite biomarkers from cfDNA using DNA microarray methodology. We isolated cfDNA from the cell-free mediums of the cultures of colorectal cancer cell lines in the presence of the control group which was the healthy epithelial colon cell line.

**Results:** Our results underlined significant alterations that were deletions and/or duplications in some of the genomic regions in a cell line-specific manner.

**Conclusion:** We propose that DNA microarray could be used to assess the sub-types of certain cancers in a non-invasive manner using cfDNA approaches.

**Keywords:** cfDNA, carcinogenesis, colorectal cancer, DNA microarray, biomarker

## INTRODUCTION

Colorectal cancer (CRC) is one of the most widespread cancers estimated by the Global Cancer Observatory listed in the 'cancer today' data of 2020 (1). CRC is in third place for the number of incidences; however, it ranks second in terms of mortality rate (2). Considering the estimated number of 1.9 million cases annually, this notorious disease devastates the economy and life expectancy worldwide. The early diagnosis of the tumorigenesis and profiling the mutations may increase the survival rate by improving the chance to fight back. It has been reported that only 30-40% of the cases were diagnosed at early stages (3). Hence,

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**Submitted:** 07.03.2023 **Revision Requested:** 24.03.2023 **Last Revision Received:** 30.05.2023 **Accepted:** 05.06.2023 **Published Online:** 04.08.2023

there is a continuous need for novel diagnostic processes to detect the disease in the initial phases.

Current screening processes for CRC involve analyzing the presence of blood in stool samples, fecal immunochemical tests and colonoscopies (4). When the result is positive, a biopsy takes place to profile the tumor. Nevertheless, the heterogeneity of cancer remains a big challenge to identify mutations (5). Testing blood samples or other body fluids could decrease the compliance of patients. Cell-free DNA (cfDNA) is one of the most promising macromolecules found in body fluids such as blood, saliva and urine (6). It originates from apoptotic, necrotic, or secretory pathways of both healthy and cancer cells of the body (4). These cleaved small DNA sequences contain biomarkers to identify and characterize heterogeneous tumor cells without blockage by tissue-specific boundaries. Therefore, cfDNA has been targeted and exploited by several studies to detect markers of cancers for early diagnosis. In these reports, next-generation sequencing (NGS), real-time polymerase chain reaction (RT-PCR) or droplet digital PCR (ddPCR) have been employed to identify the markers (7-9).

DNA microarray is a robust strategy for the identification and high-throughput screening of genomic anomalies including point mutations, chromosomal alterations such as deletions, and insertions, and copy number variations (CNVs). Microarray tools provide advantages in cancer diagnosis, classification and determination of treatment options (10, 11). Therefore, combining DNA microarray strategy with cfDNA approach would be a vigorous tactic to identify and analyze the markers embedded in cfDNA without meddling with tumor heterogeneity, tissue type and time points. Large-scale screening of cancer markers at any time interval could be possible with this robust, flexible and effortless method using only body fluids.

The present study aimed to clarify any possible markers in the cfDNAs obtained from different CRC cell lines using DNA microarray approach. Up to now, this is the first study assessing biomarker availability by combining both cfDNA and DNA microarray approaches.

## MATERIALS AND METHODS

### Cell Lines and Cell Culture

In the present study, sporadic colorectal cancer cell lines, LIM1863; colorectal carcinoma cell lines, HCT116 and CACO-2; and colorectal adenocarcinoma cell lines with epithelial morphology HT-29 were used in the presence of normal colon fibroblast cell lines CCD-18Co. All cells were grown in RPMI 1640 medium (Thermo Fischer, USA) in the presence of 1% penicillin/streptomycin (Thermo Fischer, USA) and 10% fetal bovine serum (FBS; Thermo Fischer, USA) in an incubator under 37$^0$C and 5% $CO_2$ conditions. Cells were routinely passaged when they reached 80% confluency. All experiments were performed when the confluency was 80%.

### Isolation of cfDNA

cfDNAs were isolated from the mediums in which the cells were cultured. When the cells reached 80% confluency, the medium on the cells were carefully obtained. Next, medium was slightly centrifuged at 200 g for 5 min to pellet the remaining cells. The supernatants were used for cfDNA isolation using a ZipPrime cfDNA isolation kit (ZipPrime Ltd., Turkiye). In short, 1 ml of the medium was lysed by pre-heated lysis buffer containing proteinase K (Thermo Scientific, USA). Then, the lysate was mixed by a binding solution including binding beads, and shaken for 10 min at room temperature. After mixing, the supernatants were removed with the help of a magnetic rack, and the beads were washed with washing solutions twice. Finally, the beads were dried and re-suspended with 20 µl of the elution buffer, and the supernatants containing cfDNA were obtained. The concentrations and purities of the isolated cfDNAs were determined by both NanoDrop (NanoDrop ND-1000; Thermo Scientific, USA) via optical density ratios (OD260/280 and OD260/230), and Qubit 4 Fluorometer (Thermo Scientific, USA).

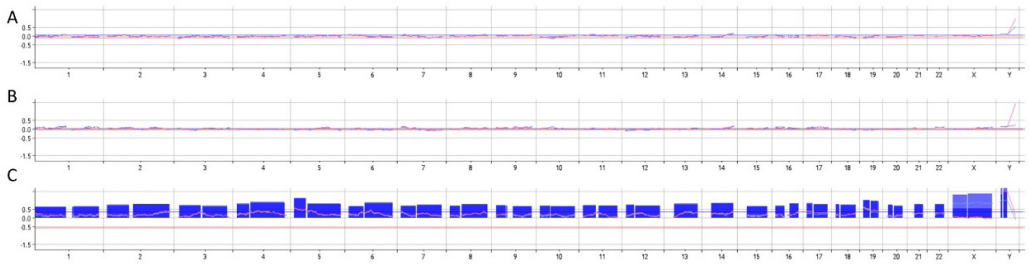


**Figure 1.** Comparison of the microarray results obtained from CCD-18Co cfDNA with the reference and assessment of genomic DNA (gDNA) contamination. The cfDNAs from CCD-18Co cells were isolated at three different culturing processes. One of them was used as the reference for all cell groups while the others (A and B) were assessed as a study group. As a further control, one of the cfDNAs was deliberately contaminated by commercial human reference DNA and a microarray was performed after whole genome amplification (WGA) step (C). Blue bars correspond to the amplifications of the regions.

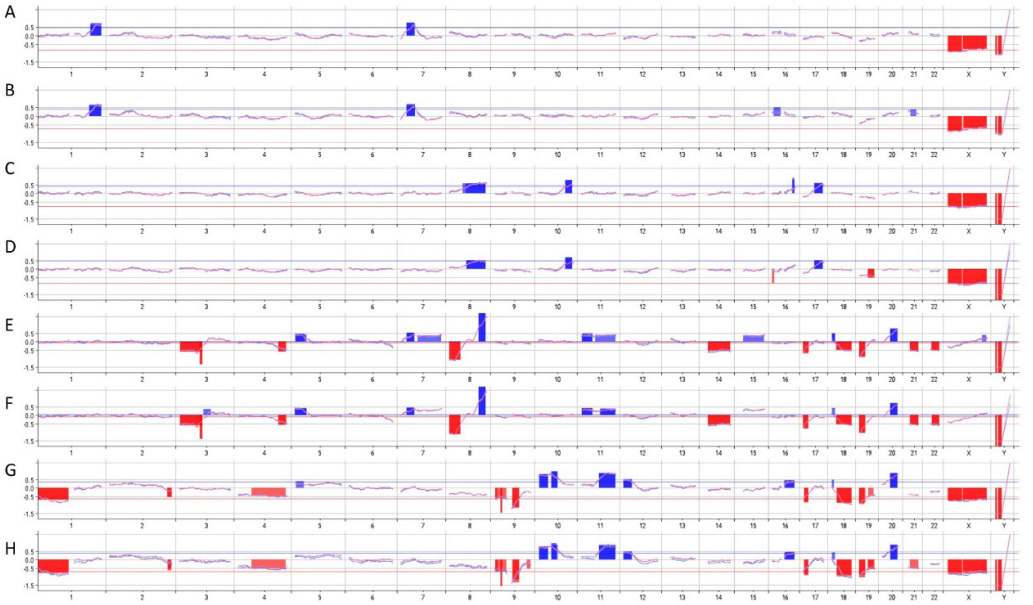


**Figure 2.** DNA microarray results from cfDNAs obtained from LIM1863 (A-B), HCT116 (C-D), HT-29 (E-F) and CACO-2 (G-H) cells. Red bars point to the losses while blue ones underline the gains.

## Whole Genome Amplification (WGA) and DNA Microarray

The isolated cfDNAs were whole-genome amplified using REPLI-g WGA kit (Qiagen, Germany) according to the supplier's instructions. The amplification was checked with 1% agarose gel. WGA products were labeled with Cyanine3 (Cy3) or Cyanine5 (Cy5) fluorophores at 37$^0$C for 2 h. After the purification step, the labeled DNAs were hybridized using GenetiSure Pre-Screen 8x60K slides (Agilent Technologies, Inc., USA) at 67$^0$C for 16 h. After the washing and drying steps, the slides were scanned by a Sure Scan Microarray Scanner (Agilent Technologies, Inc., USA). The data were analyzed by CytoGenomics Single Cell Analysis software (Agilent Technologies, Inc., USA). All annotations were according to hg19.

## Statistical Analyses

The obtaining mediums and cfDNA isolations were duplicated at different periods and thus each sample was run twice. For aberration detection, the log2 ratio was set to default as 0.35 for amplification/gain and -0.45 for deletion/loss as proposed by the application note of the system (12).

# RESULTS

## Characterization of Isolated cfDNAs

The cfDNAs isolated from the culture mediums of the cells were fluorescently quantified and the concentrations were determined between 1.51-25.3 ng/µl. The gDNA contamination was followed by deliberately addition of gDNA into one of isolated cfDNAs and performing DNA microarray by this sample. According to the results (Figure 1), possible availability of gDNA bring about remarkable copy gains compared to non-contaminated samples, underlying there were not any gDNA contamination in the study group.

## Comparison of CNVs between the cfDNAs

After the DNA microarray step, the chromosomal segments were compared to detect the common and differentiated CNVs between the cfDNAs isolated from the cell lines (Figure 2). The results underlined that some CNVs listed in the Table 1 were specific to each cell line while others were common between the cell lines as a combination of LIM1863& HT-29, HCT116& HT-29 and HT-29& CACO-2 by same loss or gain patterns (Table 2).

# DISCUSSION

CRC is one of the deadliest cancer types worldwide and the number of patients with CRC were predicted to approximately be doubled by 2040. CRC subtypes are traditionally classified according to the segment of cancer location; nonetheless, they have more complicated molecular classifications. The molecularly classified subtypes have been shown to associate with clinical outcomes. However, these subtype definitions are limited to a picture of the clinical outcome and further approaches are still needed (13).

cfDNA consists of the small DNA segments that are released by the cells into the circulatory system. In cancer, cfDNA may consist of tumor-derived DNA sequences, which could serve as a platform for biomarkers for the detection and monitoring of cancer and therapy. Numerous studies have frequently focused on cfDNA-based biomarker detection using several

**Table 1.** The differentially affected regions by gains or losses for each cell line.

| Cell line | Chr | Start-Stop | Cytoband | Size (kb) | Gain/Loss |
|---|---|---|---|---|---|
| LIM1863 | 1 | 206075579-248563836 | q32.1 - q44 | 42,488.258 | Gain |
| | 7 | 23194747-23822183 | p15.3 | 627,436 | |
| HCT116 | 8 | 67352994-142205781 | q13.1 - q24.3 | 74,852.788 | Gain |
| | 10 | 104003117-131291788 | q24.32 - q26.3 | 27,288.672 | |
| | 17 | 43120016-77220974 | q21.31 - q25.3 | 34,100.959 | |
| HT-29 | 5 | 1130129-45142098 | p15.33 - p12 | 44,011.97 | Gain |
| | 11 | 2909874-44075140 | p15.4 - p11.2 | 41,165.267 | |
| | 3 | 1464291-90273445 | p26.3 - p11.1 | 88,809.155 | Loss |
| | 8 | 215827-43407979 | p23.3 - p11.1 | 43,192.153 | |
| | 14 | 19849591-107152122 | q11.2 - q32.33 | 87,302.532 | |
| | 21 | 14420615-48022981 | q11.2 - q22.3 | 33,602.367 | |
| | 22 | 21240223-51170223 | q11.21 - q13.33 | 29,930.001 | |
| CACO-2 | 1 | 746608-121150012 | p36.33 - p11.2 | 120,403.405 | Loss |
| | 2 | 226945229-242938241 | q36.3 - q37.3 | 15,993.013 | |
| | 4 | 53656120-157605164 | q12-q32.1 | 103,949.045 | |
| | 9 | 3022488-12155545 | p24.2 - p23 | 9,133.058 | |
| | | 23915540-30045342 | p21.3 - p21.1 | 6,129.803 | |
| | | 69430459-97151185 | q21.11 - q22.32 | 27,720.727 | |
| | 10 | 3314825-38219750 | p15.2 - p11.1 | 34,904.926 | Gain |
| | | 49954828-72912411 | q11.23 - q22.1 | 22,957.584 | |
| | 12 | 792951-34285770 | p13.33 - p11.1 | 33,492.82 | |
| | 16 | 51171823-87138780 | q12.1 - q24.2 | 35,966.958 | |

**Table 2.** The same regions affected by gains or losses for the cell lines.

| Cell lines | Chr | Start-Stop | Cytoband | Size (kb) | Gain/Loss |
|---|---|---|---|---|---|
| LIM1863& HT-29 | 7 | 23822183-52965122 | p15.3 - p12.1 | 29,142.94 | Gain |
| HCT116& HT-29 | 8 | 115281701-142205810 | q23.3 - q24.3 | 26,924.11 | Gain |
| HT-29& CACO-2 | 4 | 157605164-187638862 | q32.1 - q35.2 | 30,033.699 | Loss |
| | 11 | 73712308-134790190 | q13.4 - q25 | 61,077.883 | Gain |
| | 17 | 44684-21180656 | p13.3 - p11.2 | 21,135.973 | Loss |
| | 18 | 861334-10532357 | p11.32 - p11.22 | 9,671.024 | Gain |
| | | 20663689-77954136 | q11.2 - q23 | 57,290.448 | Loss |
| | 19 | 277373-23035449 | p13.3 - p12 | 22,758.077 | Loss |
| | 20 | 31257259-60346875 | q11.21 - q13.33 | 29,089.617 | Gain |

high-throughput methods such as NGS (14-17). However, the drawbacks of the NGS systems, such as high cost and time requirements, restrict the wide applicability of the approach (18). Therefore, other high-throughput methods including the DNA microarray approach should be widespread. Huge chromosomal structural alterations and CNVs have extensively been linked to carcinogenesis, pointing out the importance of DNA microarray method in the detection and monitoring of cancer (19).

Although two phenomena, cfDNA, and DNA microarray, have been separately evaluated in cancer studies, recent efforts have focused on the combination of these approaches. For instance, Azad et al. showed the androgen receptor gene anomalies using cfDNA and DNA microarray in patients with prostate cancer (20). Similarly, the product notes of the Agilent Company presented a detailed protocol for the assessment of the power of the DNA microarray on the single-cell level (12). However, none of the studies focused on the sub-typing of specific cancer by the combination of DNA microarray and cfDNA. As a part of an ongoing study, we aimed to evaluate the efficacy of the DNA microarray method on cfDNAs isolated from the medium of the CRC cell lines; suspension sporadic colorectal cancer cell lines; LIM1863; adherent colorectal carcinoma cell lines HCT116 and CACO-2; and adherent colorectal adenocarcinoma cell lines with epithelial morphology HT-29, in the presence of adherent normal colon fibroblast cell line CCD-18Co. The properties of these cell lines, especially secretome profiles have been proven to be distinguishable at the molecular level (21, 22), underlying diversity in the genetic background. Hence, it may be possible to sub-type these cells according to differences in the cfDNA profiles.

In this study, we isolated cfDNAs from the mediums where the cells were grown. Accordingly, we removed the cells by centrifugation and used cell-free medium to obtain cfDNAs. Although we did not perform the size analyses to clearly ensure the isolation of cfDNAs, whose size was about 160 bp (16), we confirmed the lack of genomic DNA (gDNA) owing to the centrifugation step and the concentrations of isolated DNA, which were in a range of 1.51-25.3 ng/µL. Moreover, a recent study underlined that the mediums on the cells could contain mitochondria and intact mitochondrial DNA (23); therefore, a size analysis of cfDNA would not be a practical approach to prove the purity of the cfDNA. To further ensure the lack of the gDNA, additionally, we analyzed the cfDNA from the reference cell line, CCD-18Co, whose cfDNAs were separately isolated from the reference one. The involvement of gDNA in the cfDNA was proved to alter the DNA microarray results (12). However, we did not realize any differences in the CCD-18Co duplicates compared to the reference well (Figures 1A and B). This result may demonstrate the lack of gDNA contamination in the cfDNA samples. Importantly, when gDNA was deliberately contaminated into the isolated cfDNAs, the array results were completely problematic showing significant amplification of all chromosomes (Figure 1C). This result also proved that the cfDNAs were gDNA-free.

Next, we conducted a DNA microarray from cfDNAs obtained from different CRC cell lines compared to that of the reference cell line, CCD-18Co in the presence of biological controls for each cell line. According to the results, the cfDNAs had losses and gains distinguishable between the cell lines (Figure 2), which could be used to *in vitro* determine the subtypes of the CRC phenotypes. Although the amplified or lost regions were highly similar to each other, some regions (for example chromosome 16 for the HCT116 cell line); (Figures 2C and D) was drastically different between the replicas, clarifying that these regions should not be evaluated for subtyping purposes. Notably, there was a huge diversity of the sex chromosome for all cell lines. The system aims to determine the sex of the sample using sex chromosome-dependent probes. According to the American Type Culture Collection (ATCC) and The Cellosaurus databases, LIM1863 and HT-29 originate from female patients while HCT116 and CACO-2 from male ones. Nonetheless, our results did not correspond to the relative sex phenotypes. The success rate of the DNA microarray systems in properly detecting the sex chromosome has been reported to be low (24). Still, the problem with the sex chromosomes would be the result of DNA microarray application on cfDNA and requires further studies. Our propose was not about to examine the sex chromosomes in the present study.

When the common variations between the replicas were evaluated, specific affected chromosome regions were distinguishable between the cell lines. The regions were evaluated comparatively and the differentially altered regions are listed in Table 1, which could be used as a biomarker for subtyping of the CRC. Despite not being for all cell lines, the regions listed in Table 2 were common between the cell lines. Accordingly, the chromosomes of the LIM1863 and HCT116 were shown to be minimally affected while those of HT-29 and CACO-2 cells had dramatic alterations by the cfDNA screening. Chromosomal characterization by gDNA of the CRC cell lines including HCT116, HT-29, and CACO-2 has previously been carried out, and cellular and chromosomal differences were well-reported and showed the remarkable diversity between the cell lines (25), which may confirm the results of the present study. Moreover, these cell lines could be classified according to their differentiation properties and HT-29, and CACO-2 cells compared to LIM1863 and HCT116 have higher differentiation capabilities (26). For particular cell types, differentiation was shown to induce cell death (27) and the association between differentiation and cell death is well documented (28).

When the differentially affected regions (Table 1) were examined by the gene information, 656 genes were seen to have higher copies for the cfDNAs from the LIM1863 cell line. When the pathways were analyzed for these genes by Reactome Database (29), most of the gene-encoded proteins had a role in the olfactory signaling pathway that was already linked to carcinogenesis (30). For the HCT116 cell line, 1720 genes were amplified and the products of these genes mainly had a role in fibroblast growth factor receptor 2 (FGFR2) mutant receptor activation corresponding to colorectal cancer in the literature

(31). In the approximately 5.5 thousand affected genes for the HT-29 cell line, 993 genes whose products acted normally in the olfactory signaling pathway were amplified while 4654 genes whose products were involved predominantly in cellular inflammation, Fc gamma receptor (FCGR) activation and phagocytosis associating with systemic inflammatory clearance lost copies. For CACO-2 cells, similar to HT-29, about 5.5 thousand genes were affected. Of those genes, 1710 of them gained copies and the products of these genes had a role primarily in response to metal ions triggering the carcinogenesis (32), 3792 genes whose products participated in the inhibition of signaling by overexpressed epidermal growth factor receptor (EGFR) that lost copies, putatively to overcome EGFR-targeted strategies. Overall, the products of all affected genes were actualized to directly or indirectly associate with carcinogenesis as expected.

## CONCLUSION

The importance of the cfDNA has been well-documented in CRC patients (33-35). However, cfDNAs from patients and/or cell lines should be re-evaluated to diagnose, follow up or treat the CRC by combining diverse approaches. Accordingly, the present study points to the combination of the cfDNA and DNA microarray approaches to find biomarkers specific to subtypes of the CRC cell lines for the first time. Still, further studies are needed to totally explore the potential of these approaches.

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**Ethics Committee Approval:** Ethical approval is not required as the study was performed on cell lines.

**Authors' Contributions:** Conception/Design of Study – H.H.K., E.C., M.A.E.; Data Acquisition – D.B.C.; Performing experiments – C.P.K., D.B.C.; Data Analysis/Interpretation – A.C.O., H.H.K.; Drafting Manuscript – H.H.K., A.C.O., E.C.; Critical Revision of Manuscript – D.B.C., H.H.K., A.C.O., E.C.; Final Approval and Accountability– H.H.K, E.C., A.C.O., M.A.E.

**Conflicts of Interest:** The authors declare no conflict of interest.

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

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Experimed 2023; 13(2): 93-96

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1291728

# Cyclosporine Treatment Increases the ACE/ACE2 Ratio in Adipose Tissue and Aorta

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**Cite this article as:** Darilmaz SE, Bingul I, Soluk-Tekkesin M, Demir N, Bekpinar S. Cyclosporine treatment increases the ACE/ACE2 ratio in adipose tissue and aorta. Experimed 2023; 13(2): 93-96.

## ABSTRACT

**Objective:** The renin-angiotensin system (RAS) mainly functions in the regulation of vascular tone. Since the perfusion and storage capacity of adipose tissue is largely dependent on vessel density and tone, factors that modulate vasoactive RAS components may affect adipose tissue metabolism. Cyclosporine, an immunosuppressive drug, has some detrimental effects on the vascular system and kidneys by modulating the components of the RAS. However, the effect of cyclosporine on adipose tissue is still not clear. In this study, the impact of cyclosporine on mRNA expressions of some "RAS components" in the aorta and epididymal adipose tissue of rats were investigated.

**Materials and Methods:** Rats were injected subcutaneously with cyclosporine at a dose of 25 milligrams per kilogram per day for 1 week. Angiotensin II, angiotensin (1-7), blood urea nitrogen and creatinine levels were examined in serum samples of rats. mRNA expressions of RAS components in the aorta and epididymal adipose tissue, and hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) in epididymal adipose tissue were determined. In addition, histopathological examinations of the adipose tissue were performed.

**Results:** Cyclosporine administration significantly suppressed the expression of angiotensin-converting enzyme 2 (ACE2) in both the aorta and adipose tissue. Accordingly, it caused a significant increase in the ACE/ACE2 ratio. However, it did not affect the adipose tissue HIF-1 and VEGF expressions. When examined histopathologically, no change was observed in the vascular density of adipose tissue due to cyclosporine.

**Conclusion:** It was thought that the increase in the ACE/ACE2 ratio might be an adaptation that protects the adipose tissue against the systemic effect of cyclosporine.

**Keywords:** Cyclosporine, adipose tissue, angiotensin-converting enzyme 2, vascular density

## INTRODUCTION

Cyclosporine is an immunosuppressant medication used in clinical practice to prevent organ graft rejection and to treat autoimmune diseases. However, this drug shows additional effects on non-immune cell populations and may adversely affect the vascular system and kidneys (1).

It has been reported that upregulation of the vasoconstrictive renin-angiotensin system (RAS) and endothelin-1, inhibition of nitric oxide (NO)-dependent vasodilation, and also increasing the formation of free radicals are effective in the hypertensive and nephrotoxic effects of cyclosporine (1).

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**Submitted:** 03.05.2023 **Revision Requested:** 24.05.2023 **Last Revision Received:** 25.05.2023 **Accepted:** 05.0.2023 **Published Online:** 26.07.2023

**Table 1.** Serum parameters of control and cyclosporine-treated rats.

| | CONTROL (n=7) | CYCLOSPORINE (n=9) |
|---|---|---|
| Ang-II (pg/mL) | 66 ± 2.7 | 61 ± 5.0 |
| Ang 1-7 (pg/mL) | 59 ± 1.6 | 48 ± 3.1 [a] |

**Note:** Values are means ± SEM. In statistical evaluations, a Mann–Whitney U test were used.The unpaired Student's t-test was used for equal variances. Ang-II, angiotensin II; Ang (1-7), angiotensin (1-7).
[a]: $p < 0.05$ compared with the control group.

**Table 2.** Vascular density score in epididymal adipose tissue in control and cyclosporine-treated rats.

| | Vascular density score |
|---|---|
| Control group | 10.83 ± 0.74 |
| Cyclosporine-treated group | 12.25 ± 0.62 |

**Note:** Values are mean± SEM (*n* = 7–9 in each group). Vessels in three different areas of adipose tissue section of each rat were counted and averaged. In statistical evaluations, a Mann–Whitney U test were used.

Angiotensin II (Ang-II), which is synthesized by catalysis of angiotensin-converting enzyme (ACE), exhibits a vasoconstrictive effect when it interacts with the Ang-II type-1 receptor (AT1R), whereas a vasodilator effect is observed by interacting with the Ang-II type-2 receptor (AT2R). Moreover, angiotensin (1-7), synthesized by angiotensin-converting enzyme-2 (ACE2) enzymatic activity, has a vasodilator effect when it binds to the Ang (1-7) receptor (Mas R). The antagonistic effects of RAS components are crucial in the regulation of vascular tone and perfusion of the relevant organ.

RAS components also exert a crucial role in adipose tissue metabolism in an autocrine or paracrine manner (2,3). It has been reported that RAS components with vasoconstrictive effects are largely responsible for hypertension that seen in obese individuals (2, 3).

Although its apparent effects on the vascular system and kidneys are known, how cyclosporine affects adipose tissue metabolism has not been studied much. In a study administering to rats by gavage for six weeks, cyclosporine was reported to decrease glucose uptake into adipocytes, increase lipolysis and suppress lipogenesis (4).

In this study, we aimed to elucidate the effect of cyclosporine administration to rats for 1 week in mRNA expressions of RAS components in both epididymal adipose tissue and the aorta. In addition, we also elucidated the vascular density and the mRNA expressions of HIF-1 and VEGF in adipose tissue.

## MATERIALS AND METHODS

### Animals

In this study, sixteen male Wistar rats weighing approximately 350 grams, provided by the Bezmialem University, Experimental Medical Research Institute were housed in a stainless cage (three to four per cage) with temperature and light control (12 h dark/12 h dark) and received a standard pellet diet. The experimental procedures were implemented according to the order of Bezmialem University, Animal Experiments Local Ethics Committee (Date: 28.02.2019, No: 33).

### Experimental Design

Cyclosporine group (n=9): Cyclosporine A (Sandimmune, Novartis, Switzerland) was prepared in ethanol (94%) and polyoxyethylated castor oil and a dose of 25 mg/kg was applied subcutaneously once daily during the one-week experimental period, according to previous studies (5).

Control group (n=7): Rats were exposed to daily subcutaneous injections of Cremophore EL: alcohol (2:1) as a vehicle during the one-week experimental period.

### Samples

Rats were given ketamine (35 mg/kg, Pfizer, USA) and xylazine HCl (15 mg/kg, Bioveta, Czech Republic) intraperitoneally for anaesthesia, and euthanized after an overnight fasting. Blood samples were taken from their hearts into tubes; thereafter, sera were obtained by centrifugation.

The thoracic aorta and epididymal adipose tissue samples were isolated from each rat.

### Determination of Ang-II, Ang (1-7) , Blood Urea Nitrogen (BUN) and Creatinine in Serum

Ang-II and Ang (1-7) (Rat ELISA kit; Ang II #101212; Ang (1-7) #101211; Abbkine, Inc., China), as well as BUN and creatinine (Roche Cobas auto-analyzer) levels in sera were determined.

### Determination of mRNA Expressions in the Aorta and Adipose Tissue

AT1R, ACE, and ACE2 mRNA expressions in the aorta and ACE, ACE2, HIF-1 and VEGF mRNA expressions in the epididymal fat sample were measured. A commercial kit (NucleoSpin RNA Isolation Kit, #740955, Macherey-Nagel, Germany) was used for the RNA purification. Following that procedure, 5 ng purified RNA was also used to synthesize the cDNA samples (cDNA Synthesis Kit; #PCR511, Script, Jena-Bioscience GmbH, Jena, Germany). After the cDNA acquisition, quantitative real time-polymerase chain reaction (qRT-PCR) was carried out by using the qPCR Sybr Green Master Mix kit from Jena Bioscience (#PCR372, GmbH, Jena, Germany) with the following primers of AT1R (NM_004835, F:5'-TTCACCCTGCCTCAGGATCT-3',

R:5'-CCAGACCCACCAATCCA TCC-3');

ACE (NM_000789, F:5'-CGTCCACCGTTACCAGACAA-3',
R:5'-TTGGCCTCTGCGTATTCGTT-3');
ACE2 (NM_021804, F:5'-GAATGCGACCATCAAGCGTC-3',
R:5'-CAAGCCCAGAGCCTACGATT-3');
VEGF (NM_001025366, F:5'-GCACTGGACCCTGGTTTAC-3',
R:5'-GGGTCTCAATTGGACGGCAA-3') and
HIF-1 (NM_001530, F:5'-ATGTACCCTAACAAGCCGGG-3',
R:5'-AAGCACGTCATAGGCGGTTT-3') (LGC Biosearch Technologies, Denmark) in a Biorad-CFX Connect device (California-USA). The relative mRNA expressions were calculated with the method of $2^{-\Delta\Delta CT}$. The expression of mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an internal control, was used to adjust the analytical data.

## Histopathological Examination

Samples of epididymal adipose tissue were preserved in a 10% buffered formalin solution, which was then paraffin blocked. Hematoxylin-eosin stained preparations were observed morphologically under a light microscope. For scoring of the vascular density, three different areas were chosen, and then the number of vessels was counted and vascular density was calculated by dividing the total number of vessels by three.

## Statistical Analyses

The statistical analyses were implemented by using the Statistical Package for The Social Sciences (21.0; SPSS Inc., Chicago, IL-USA) program, unpaired Student's ttest or Mann-Whitney Utest, according to data distribution. Correlations were evaluated by Pearson's test. When p-values are less than 0.05, they were considered significant.

## RESULTS

Cyclosporine administered daily for one week into animals at a dose of 25 mg/kg, did not affect renal function; BUN and creatinine levels in serum did not change ( Cyclosporine: 24.7 ± 1.1 mg/dL - Control: 25.6 ± 4.7 and Cyclosporine: 0.36 ± 0.01mg/dL - Control: 0.31 ± 0.03 respectively). While serum Ang- II levels were not affected by cyclosporine treatment, Ang (1-7) levels decreased (Table 1).

Cyclosporine treatment in aortic tissue significantly increased the mRNA expression of AT1R. However, a significant decrease in ACE2 expression and an increased ACE/ACE2 ratio were observed (Figure 1).

A significant cyclosporine-induced decrease in ACE2 expression was also found in adipose tissue, resulting in a marked increase in the ACE/ACE2 ratio. However, AT1R and ACE expressions remained unchanged with cyclosporine treatment (Figure 2). To evaluate the effect of cyclosporine on adipose tissue perfusion, we examined histopathologically the vascular density and RNA expressions of HIF-1 and VEGF. We did not detect any change in these parameters due to cyclosporine (Figures 2 and 3, Table 2).

But interestingly, a significant negative correlation was found between ACE/ACE2 ratio and HIF-1 in adipose tissue (r= -0.651, p=0.009).

## DISCUSSION

The effect of the administration of 25 mg/kg cyclosporine to rats for one week on mRNA expressions of RAS components in the aorta and epididymal adipose tissue were investigated in the current study. In the aorta, this immunosuppressive drug caused an increase in AT1R and a decrease in ACE2 expression and a marked increase in the ACE/ACE2 ratio. In addition,

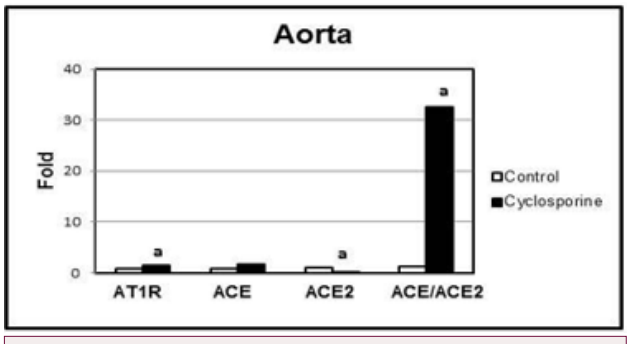


**Figure 1.** The impact of cyclosporine on mRNA expressions of RAS components in rat aorta

Values are given as mean ± SEM (*n*=7-9 for each group).

a: p < 0.05; compared to the control. AT1R, angiotensin-II type-1 receptor; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2

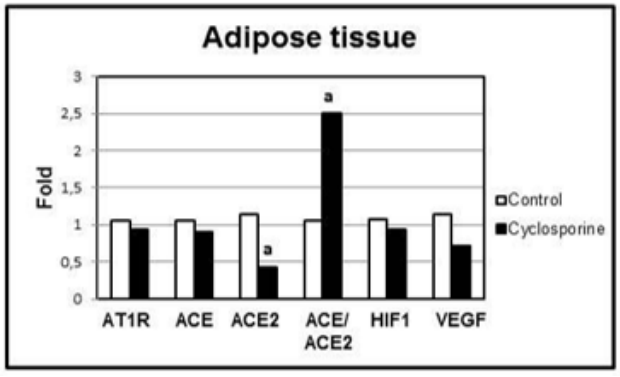


**Figure 2.** The impact of cyclosporine on mRNA expressions of RAS, HIF-1 and VEGF in rat epididymal adipose tissue

Values are given as mean ± SEM (n=7-9 for each group).

a: p < 0.05; compared to the control. AT1R, angiotensin-II type-1 receptor; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2, HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor

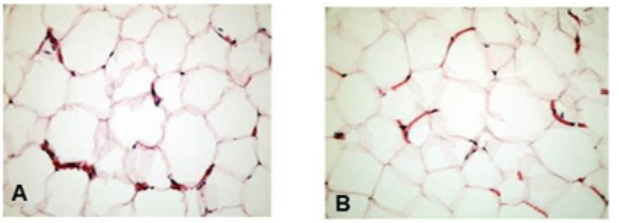


**Figure 3.** The representative images of the epididymal adipose tissue stained by hematoxylin-eosin (n=7-9 in each group). Control (A) and cyclosporine (B) groups have a similar appearance. Thin vessel sections were observed between adipocytes. (Original magnification×400)

serum Ang-II levels remained unchanged, but Ang (1-7) levels were found to be low in the cyclosporin group. Our findings showed that at least in part that cyclosporine has a systemic vasoconstrictive effect, as previously described (1, 6, 7).

Adipose tissue is a highly vascularized organ. Beyond supplying oxygen and nutrients, the vascular system carries a variety of cytokines, growth factors, and hormones to and from adipose tissue. Any modulation of vessel density and vessel diameter of adipose tissue affects tissue perfusion and function. The main transcription factor regulated by oxygen homeostasis and hypoxia is HIF-1. Under hypoxic conditions, HIF-1 activates the transcription of genes facilitating metabolic adaptation to hypoxia such as glycolytic enzymes, vascular endothelial growth factor, etc. (8)

In our study investigating the effect on some vasoactive RAS components and hypoxic parameters in adipose tissue, it was found that cyclosporine decreased ACE2 expression and increased the ACE/ACE2 ratio. However, HIF-1 and its target gene VEGF expressions were not affected by this drug. Furthermore, no change was observed histopathologically in the vascular density of adipose tissue due to cyclosporine. But interestingly, a marked inverse association was detected between the ACE/ACE2 ratio and HIF-1 in adipose tissue.

It is well known that the increase in systemic blood pressure passively dilates the vessels in the resistant arteries and arterioles embedded within the parenchyma. To compensate for this expansion, the smooth muscle cells of the vessel contract and try to return the vessel diameter to its initial state. This reactive contraction, called the myogenic response, counteracts the effect of a change in systemic pressure on tissue perfusion (9, 10). Multiple mechanisms have been reported to mediate the myogenic response depending on their location in the body (9). In a study conducted in cerebral resistant arteries, the role of AT1R activation in the pressure-induced myogenic response was indicated (11).

According to our findings, the increase in ACE/ACE2 ratio observed due to cyclosporine in our study may be related to the myogenic adaptation that protects the adipose tissue against the systemic effect of cyclosporine.

**Ethics Committee Approval:** This study is approved by Bezmialem University, Animal Experiments Local Ethics Committee (Date: 28.02.2019, No: 33).

**Authors' Contributions:** Conception/Design of Study – S.B, I.B.; Data Acquisition – S.B., I.B.; Data Analysis/Interpretation – S.B., I.B., E.D.; Drafting Manuscript– I.B.; Critical Revision of Manuscript- S.B.; Final Approval and Accountability– I.B.; Technical or Material Support- E.D., I.B.; M.S.T, N.D.; Supervision- S.B.

**Conflicts of Interest:** The authors declare no conflict of interest.

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

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Experimed 2023; 13(2): 97-102

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1285692

# Characterization of Rubidium-Based Nanoparticles by Green Synthesis and Their Effect on Colorectal Cancer Cells

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**Cite this article as:** Ozerkan D, Kariper IA. Characterization of rubidium-based nanoparticles by green synthesis and their effect on colorectal cancer cells. Experimed 2023; 13(2): 97-102.

## ABSTRACT

**Objective:** Colorectal cancers pose a major threat along with increasing morbidity and mortality to human health worldwide. Therefore, it is crucial to develop effective and safe methods for tumor therapy. In recent years, nanoparticles have emerged as successful candidates for drug delivery into tumor tissues. The particle size of nanoparticles (NPs) is of great importance for passive tumor targeting. Therefore, in this study, we aimed to synthesize and characterize rubidium-based nanoparticles (RbNPs) from the moss *Abietinella abietina* (AA) and determine their anticancer effects on colorectal carcinoma cell line (HCT116).

**Materials and Methods:** A field emission scanning electron microscope (FESEM), dynamic light scattering (DLS), energy dispersive X-ray analysis (EDX), UV/VIS and fourier transform infrared (FTIR) spectrophotometers were used to characterize the RbNPs. To study the cytotoxicity, a sulforhodamine B (SRB) assay was performed in colorectal carcinoma cell cultures.

**Results:** As a result, RbNPs- AA developed with an average particle size of about 70 nm. RbNPs- AA proved to be cytotoxic at lower doses than free AA, as it decreased cell viability at half the amount of free AA (14.25 µg/mL).

**Conclusion:** The availability of RbNPs, particularly for the treatment of colorectal cancer, is evidenced by the fact that all the data collected are highly relevant.

**Keywords:** Colorectal cancer, rubidium nanoparticles, green synthesis

## INTRODUCTION

Besides gastrointestinal cancers, colorectal carcinoma (CRC) is the third most common malignancy and the third leading cause of cancer-related deaths worldwide (1). The most common therapies for CRC include surgery, chemotherapy and radiotherapy for all stages of the disease, and adjuvant and neoadjuvant therapy for advanced stages (2). The fact that symptoms follow a similar course to other bowel diseases and that CRC is not diagnosed until the 3rd and 4th stages, with no response to the treatments used or resistance to treatment, reduces the survival rate for this disease.

Although many alternative methods have been explored, progress is still in its infancy. Tumor-targeted nanostructure systems are therefore heavily involved in cancer research studies (3, 4). In the last 20 years, the environmentally friendly production of chemicals for a sustainable future has been the subject of extensive research worldwide. The choice of an environmentally friendly solvent, an effective reducing agent, and an effective stabilizing agent are the three most important requirements for the production of nanomaterials. However, the biosynthetic method is considered a safe and environmentally friendly green method for the synthesis of nanoparticles for medical purposes. Generally, the chemical methods

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**Submitted:** 19.04.2023 **Revision Requested:** 16.05.2023 **Last Revision Received:** 27.05.2023 **Accepted:** 14.06.2023 **Published Online:** 28.07.2023

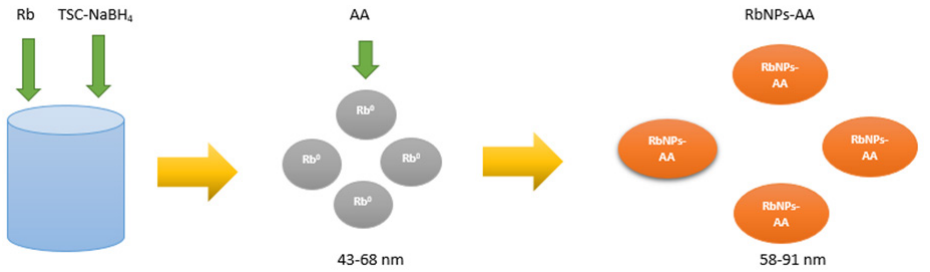



**Diagram 1.** Preparation of RbNPs and RbNPs-AA

used are expensive and involve the use of hazardous and harmful chemicals that pose many risks to the environment (4). Researchers around the world are constantly working on environmentally friendly production techniques that use green nanotechnology to develop highly effective, non-toxic, and environmentally safe products (5). Nanocarriers used in biology and medicine should have low or no toxicity and be easy to integrate and degrade (6). Metallic nanoparticles can easily cross biological barriers and penetrate into human tissues through the pores of the skin. They can bind to proteins and nucleic acids, be absorbed into cell membranes, enter the nucleus and alter the activities of cells, and exhibit greater cellular activity due to their small size and large surface area (7, 8).

The oldest plants, called bryophytes, which can be used in green synthesis, can adapt to a wide range of environmental conditions. They generally have inadequate growth and defense systems. Therefore, liverworts depend on the metabolic processes of secondary metabolites for both survival and environmental protection (9). In addition, some components of the mosses inhibit tumor growth and show an anticarcinogenic effect on various cancer cell lines (10). *Abietinella abietina* (AA) is a moss species from the Thuidiaceae family (11). The discovery of the antileukaemic activity of P-388 in the ethanol extract of *Claopodium crispifolium*, which belongs to the Thuidiaceae family, led to more mosses being collected and studied in 1980 and 1981 (12). Recently, in our work, we investigated different mosses with different extraction solutions, including the cytotoxic effect of AA on the 5-fluorouracil (5-FU) selected CD24+ colon cancer cells. In comparison to many different extracts of AA, the ethanol extract showed the highest cytotoxicity and decreased the Rho123 low cell population in 5-FU resistant HCT116 colon cancer cells (13). Therefore, we wanted to continue this study and analysed the efficacy of ethanol extract of *i* with a nanocarrier at this time. For this reason, rubidium metal-based nanoparticles (RbNPs) were synthesized using AA moss by green synthesis technique and their effects on colon cancer cells (HCT116) were evaluated after their characterization in this study.

## MATERIALS AND METODS

Rubidium dinitrate ($Rb(NO_3)_2$), Sodium borohydride ($NaBH_4$) and trisodyum nitrate ($Na_3C_6H_5O_7$) (Sigma Aldrich) were used for RbNPs. $NaHCO_3$ (Sigma), Dulbecco's modified eagle medium (DMEM; Sigma), dimethyl sulfoxide (DMSO) (Sigma) and fetal bovine serum (FBS) were used for cell culture assays.

### Liverworth Extraction

AA was freeze-dried, and its extracts were obtained. Absolute ethyl alcohol, which has a boiling point of 78.37 °C and a relative polarity of 0.654, was utilized as the dissolvent. A sample-to-solvent ratio of 1:50 was used in the extraction. All of the process was done in the Soxhlet apparatus. After 24 h extraction, the solutions were completely evaporated in a rotary evaporator (Heidolph) at 40°C under a vacuum. For analysis, the powdered dry extracts were diluted with DMSO.

### Preparation of RbNPs and RbNPs-AA

Stock solutions of 0.1 mM $Rb(NO_3)_2$, 4.3 x $10^{-3}$ M $Na_3C_6H_5O_7$, and 2 x $10^{-3}$ M $NaBH_4$ were prepared. First, 2 mL of $Rb(NO_3)_2$ was mixed with 24 mL of $Na_3C_6H_5O_7$ and 24 mL of $NaBH_4$ for 15 min. at 60 °C. The mixture was then shaken for an additional 15 min. at 90 °C. The obtained RbNPs stock solution was diluted with the extract of 1 mg/mL AA prepared in an aqueous medium (Diagram 1).

### Dynamic Light Scattering (DLS) and Zeta Sizer Analysis

Zetasizer measurements were performed at 22-23°C DLS evaluation with the Zetasizer Nano ZS using a 4 mW He-Ne laser at a wavelength of 633 nm and a detection angle of 173 °C.

### Energy Dispersive X-Ray (EDX)-Field Emission Scanning Electron Microscope (FESEM) analysis

RbNPs and RbNPs-AA samples were trickled onto a glass substrate and covered with Au/Pd at a thickness of 450 mm using a small Polaron sc 7620 sputter coater, then analysed by FESEM and EDX. To image liquid samples for analysis, samples were dropped onto glass substrates, dried, and then placed on the instrument.

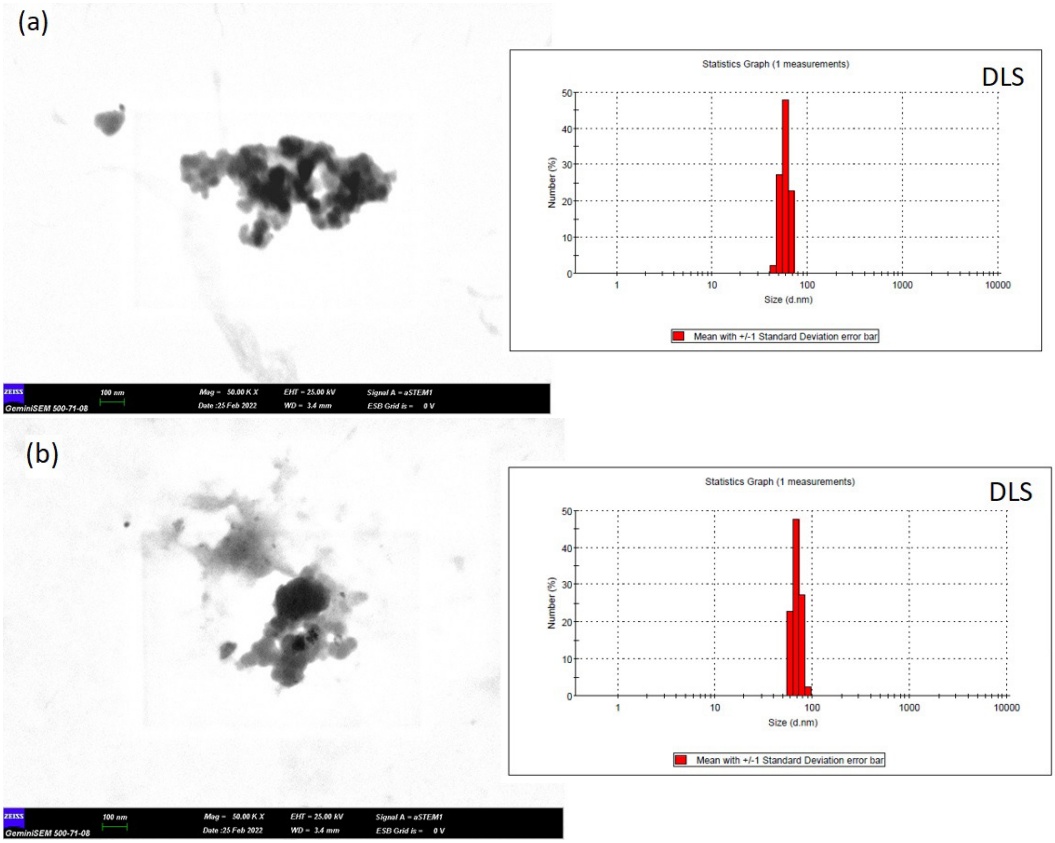



**Figure 1.** FESEM images and DLS particle size distribution of (a) RbNPs and (b) RbNPs-AA.

## Fourier Transform Infrared (FTIR) Analysis of RbNPs and RbNPs-AA

FTIR analyses of the samples were performed using a Perkin Elmer Spectrum (400 FT-IR/FT-FIR Spectrometer Spotlight 400 Imaging System).

## Absorption Measurements

The Hach Lange DR 5000 spectrophotometer was used to evaluate the absorbance of aqueous solutions of plastics. Water was used as a reference for measurements in the wavelength range 200-1100 nm.

## Preparation of Colorectal Carcinoma Cell Line

A total of 1x10$^5$ cells/mL (ATCC® CCL-247™) cells were cultured in DMEM containing 10% FBS and 1% penicilin/streptomycin and incubated at 37 °C in an incubator containing 5% $CO_2$ for 48 hours.

## Effects of AA and RbNPs-AA on the Cell Viability on HCT116 with Sulforhodamine B (SRB) Assay

SRB is a fluorescent dye that is used to quantify cellular proteins in cultivated cells as well as laser-induced fluorescence. The SRB test determines the volume of all proteins and connects it with the number of cells based on the dye's affinity for amino acids in proteins in cells (14). For this aim, lyophilized extract stock solutions were prepared in DMSO and then diluted with six different doses of free AA and RbNPs-AA (114, 57, 28.5, 14, 25,

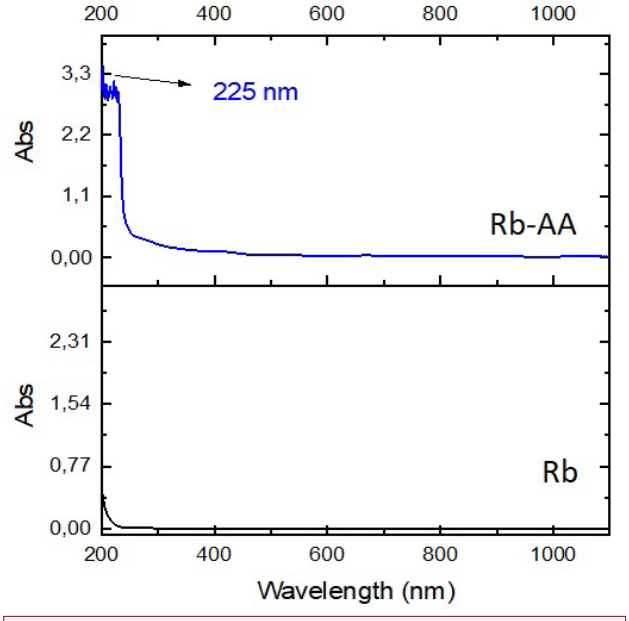



**Figure 2.** UV-VIS measurement results of RbNPs.

7.125, and 3.56 µg/mL) before being administered to HCT116 cells and cultured for 48 hours at 37°C in a 5% $CO_2$ incubator. After 48 hours, the antiproliferative effect of free AA and RbNPs-AA against colon cancer cells was performed. Dose-response curves were generated to determine the $IC_{50}$ of a compound (the concentration that prevents 50% of cells from growing). Using the Graphpad Prism 5 tool, the efficacy of the extracts was compared using this metric.

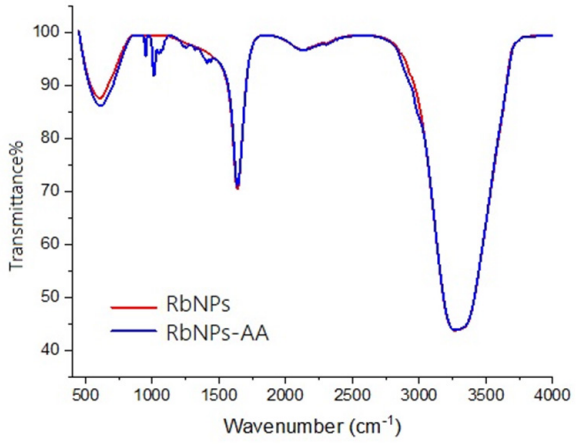



**Figure 3.** The FTIR data of RbNPs, and RbNPs-AA extract.

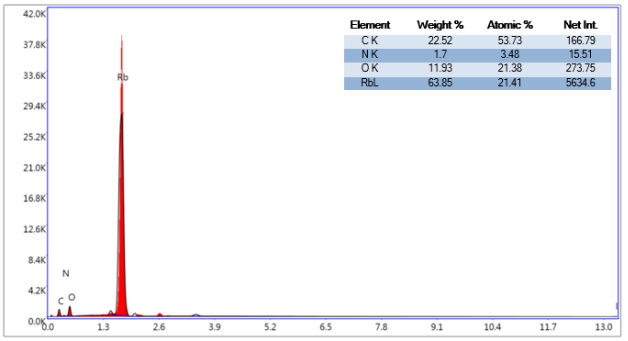


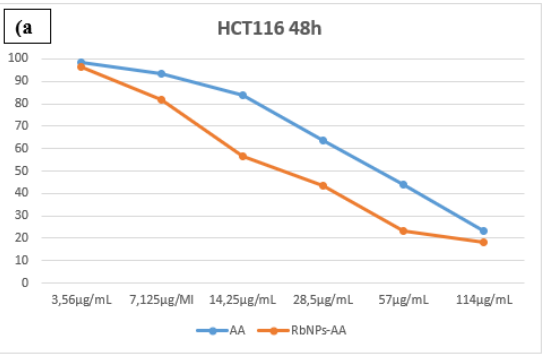

**Figure 4.** EDX analysis of RbNPs-AA.

## RESULTS

In the present work, the size distributions of two RbNPs are compared. The RbNPs produced ranged from 43.82 nm to 68.06 nm, as shown by the DLS data in Figure 1. The approximate particle size is 58 nm on average. The particle diameters of the AA extract linked to RbNPs varied from 58.77 nm to 91.28 nm. FESEM evaluation shows that although the nanoparticles stick together after solvent evaporation, particles between 50 nm and 60 nm are easily visible. In contrast, RbNPs-AA extracts discolored the soil and made it difficult to distinguish particles below 100 nm (Figure 1). This is due to the fact that the amine groups in the extract structure of AA contain dye features.

In the studies of UV-VIS, no pronounced RbNPs absorption peak was detected in the visible spectrum. However, in the composition with the AA extract, strong peaks were detected near the ultra-UV region of 205-225 nm (Figure 2).

In Figure 3, the first peak was shown at 3276 $cm^{-1}$ for the -OH band, the second peak was measured at 1645 $cm^{-1}$ for the carboxylate group of $Na_3C_6H_5O_7$, the band for -CH vibrations at lower wavelengths. Shifts were observed for RbNPs-AA compared to RbNPs. In the RbNPs-AA extract, the -OH vibrational band shifted from 3276 $cm^{-1}$ to 3242 $cm^{-1}$ due to the amine groups in the structure. Moreover, characteristic vibrational peaks such as -CN stretching, -OH bending, -CH bending, -CO stretching between 1634-1007-934 $cm^{-1}$ were measured in the RbNPs-AA group.



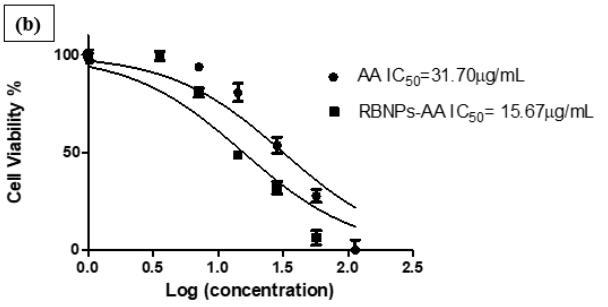




**Figure 5a.** The results of cell viability percentages of RbNPs-AA and free AA. **b.** $IC_{50}$ values of RbNPs-AA and free AA.

AA extract had 53.73% carbon, 3.48% nitrogen and 21.38% oxygen, while RbNPs contained 21.41% Rb according to EDX analysis. A very high percentage of the carrier RbNPs was found in the structure (Figure 4).

The effects of nanoparticles on cancer cell viability are determined by cytotoxicity analysis. In this way, the effectiveness of nanoparticles on cells can be better understood. Therefore, the SRB assay was used in this study, which is the preferred cytotoxicity assay. Accordingly, RbNPs-AA inhibited cell viability at half the dose of free AA (14.25 µg/mL) (Figure 5a). When $IC_{50}$ values were compared, RbNPs-AA was found to be more cytotoxic (Figure 5b).

## DISCUSSION

To prepare nanoscale pharmaceutical preparations, anticancer drugs have recently been combined with nanoscale matrix materials by covalent chemical bonding, non-covalent chemical interactions (15), encapsulation by hydrophobic interactions, and encapsulation. The water solubility of drugs, distribution in tissues, and *in vivo* pharmacokinetics can be altered using matrix materials, and absorption in cells is also significantly enhanced (16). As a key factor in each stage of nanoparticle packaging, particle size is a crucial characteristic of nanoparticles (17). According to studies (18, 19), small nanoparticles have the advantage of penetrating into tumor tissues, while larger nanoparticles can passively enter the blood and aggregate near tumor blood vessels due to their increased permeability and retention (EPR). The presence of an extracellular matrix (ECM) and anomalous interstitial fluid pressure (IFP) have also been discovered as physiological barriers that contribute to osmotic barriers (20). The force that drives the diffusion of nanomedicines from blood vessels into the tumor is undermined by increased IFP in the tumor, resulting in a lower pressure differential between them (21). A thick barrier that restricts the passage of nanomaterials to the tumor core is formed by the ECM, which includes very active macromolecules that envelop cancer tissue (20). Therefore, it is inconvenient for large nanoparticles to pass through blood vessels and reach the distal end of cancer cells, especially in desmoid tumors that are enclosed by the thick tumor tissue and lack of blood vessels. In another experiment, it was found that 300 nm liposomes enhance the efficacy of surface modification but do not enter the tumor as well as 100 nm liposomes. Clearly, the small particle size of the nanocarrier contributes to a longer half-life of the drug and better tumor penetration. In addition, tumors are prone to collect nanoparticles that have substantial permeability and are below 70 nm in size (22). The average particle size of RbNPs-AA is 70 nm. Thus, the nanoparticle sizes found in this work are quite perfect. In addition, it is possible to determine the size of the particles using XRD patterns and the change in band gap absorption in the UV-VIS spectrum. The strong peaks of RbNPs-AA near the ultra-UV region of 205-225 nm is due to the absorption peaks of the electronic -* transitions in the AA -extract structure originating from the carboxyl and amine groups (23). FTIR analysis uses infrared beam to scan samples to describe inorganic, organic, and molecular components. A change in chemical structure can be easily detected by changes in the typical pattern of absorption bands. FTIR is useful in defining and identifying unidentified compounds, detecting additions, finding impurities in a substance, and detecting degradation and oxidation (24). While the characteristic vibrational peak of RbNPs was seen at 596 $cm^{-1}$, it was simultaneously found that this vibrational signal shifted to 607 $cm^{-1}$ after AA extraction charge (25). On the other hand, for a thorough chemical evaluation of nanoparticles, EDX has proven to be an effective and quite fast characterization technique (26). In this study significant results were detected by EDX analyzes.

Currently, there are very few studies on the preparation of RbNPs. Rubidium chloride (RbCl)-doped magnesium oxide (MgO) nanoparticles were prepared by Suba et al. (27), using grape juice, and they discovered that these RbNPs have antibacterial properties. A new class of Rb-containing bioactive glass nanoparticles (Rb-BGNs) with particle sizes below 100 nm was prepared in another study, and the results suggested that Rb-BGNs could be effective bioactive fillers for bone regeneration applications (28). According to Khorshid et al. (29), PMF, cesium, and rubidium nanoparticles were found to increase apoptosis in A549 lung cancer cells.

## CONCLUSION

In summary, we have synthesized very small RbNPs with AA extract. The green synthesis approach has remarkable properties suitable for anticancer purposes. This is because the lower dosage of RbNPs-AA, which we used for this study in contrast to the free AA group, proved to be two-fold cytotoxic to colon cancer cells. Bryophytes are plants that do not have to be grown laboriously all over the world, are abundant and contain a lot of bioactive substances. Therefore, they are of great importance for anticancer drug/active substance content. Furthermore, very detailed studies on rubidium nanocarriers have not yet been carried out. So, the efficacy of RbNPs prepared in our work using the green synthesis approach will serve as a model for further studies. Besides, the isolation of biologically active substances from moss extracts could be useful in further investigations of agents providing protection against colorectal cancer.

**Acknowledgement:** The *Abietinella abietina* moss specimens used in the study were obtained from the personal herbarium of Kerem Canlı. We would like to thank him for his invaluable input and support throughout the research process.

**Ethics Committee Approval:** Ethics committee approval is not required for cell culture studies in the article.

**Authors' Contributions:** Conception/Design of Study – D.O., I.A.K.; Data Acquisition – D.O., I.A.K.; Data Analysis/Interpretation – D.O., I.A.K.; Drafting Manuscript– D.O., I.A.K.; Critical Revision of Manuscript- D.O., I.A.K.; Final Approval and Accountability– D.O., I.A.K.

**Conflict of Interest:** Authors declared no conflict of interest.

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

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Experimed 2023; 13(2): 103-108

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1284215

# Comparison of Gene Expression Levels in the p53 Pathway in Blood and Bone Marrow of Healthy Individuals

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**Cite this article as:** Daglar Aday A, Oztan G, Suer I. Comparison of gene expression levels in the p53 pathway in blood and bone marrow of healthy individuals. Experimed 2023; 13(2): 103-108.

## ABSTRACT

**Objective:** A bone marrow (BM) sample is largely used in the diagnosis and prognosis follow-up of many hematological malignancies. BM aspiration is a more risky and laborious technique compared to blood collection. Together with publications in which the expression levels in BM and peripheral blood (PB) are correlated for many genes, there are also conflicting publications. This may also be due to the physiological and disease state. In this study, we aimed to compare the BM and PB expression levels of genes in the p53 pathway in healthy individuals.

**Materials and Methods:** The study comprised 23 healthy individuals. The expressions of 22 genes in the p53 pathway were analyzed using the RT$^2$-profiler polymerase chain reaction (PCR) array. The expression levels were normalized to the reference gene β-actin. Then the mRNA expression levels between PB and BM sample groups were compared.

**Results:** The expression levels of the 20 genes studied were similar between the two groups. Only *GADD45* and *PTX3* genes were differentially expressed between PB and BM sample groups (p=0.003 and p=0.033, respectively) and those two gene expression levels were strongly correlated (r=0.886, p<0.0001).

**Conclusion:** When the expressions of 20 genes other than the GADD45 and PTX3 in our panel were evaluated, we suggest that PB largely reflects the p53 pathway gene expression levels in the BM. Therefore, PB may be preferred as an alternative to invasive BM in the analysis of these 20 genes in patients with hematological malignancies.

**Keywords:** p53 pathway, bone marrow, peripheral blood

## INTRODUCTION

The canonical functions of p53 in cell division, DNA repair, cellular senescence, and cell death are well-known (1). Recent studies have shown that wild-type and mutant p53 are involved in amino acid, nucleotide, and lipid metabolism, as well as other major metabolisms such as oxidative phosphorylation, glycolysis, and redox homeostasis (2, 3). From a metabolic point of view, the p53 pathway should be considered as a non-linear pathway. Furthermore, the complex metabolic network controlled by p53 regulators and the function of p53 in these mechanisms is not fully understood (3).

Peripheral blood (PB) material includes plasma and blood cells (erythrocytes, platelets, leukocytes). Apart from blood cells, plasma contains sugar, fat, salt, and water. Blood carries nutrients and oxygen to the cells, as well as removing waste from the cells (4). PB is used in many diagnostic tests because it can be collected less invasively than most other

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**Submitted:** 16.04.2023 **Revision Requested:** 23.05.2023 **Last Revision Received:** 26.05.2023 **Accepted:** 16.06.2023 **Published Online:** 07.08.2023

body materials and obtaining blood is both easier and less risky compared to bone marrow (BM) collection (5). BM is localized within the bone and is responsible for the production of blood cells. Hip and scapula bones are frequently preferred sites for

**Table 1.** Comparison of expressions of p53 pathway genes between bone marrow and peripheral blood samples.

| *Gene* | Groups | Number of participants | Relative expression (mean ± SD) | Fold change | p-value |
|---|---|---|---|---|---|
| *BAX* | BM | 8 | 0.3273 ± 0.91941 | 1.15 | 0.353 |
| | PB | 15 | 0.0036 ± 0.00072 | | |
| *CDKN2A* | BM | 8 | 0.0005 ± 0.00138 | 0.72 | 0.330 |
| | PB | 15 | 0.0000 ± 0.00001 | | |
| *APAF1* | BM | 8 | 0.0084 ± 0.01071 | 1.94 | 0.456 |
| | PB | 15 | 0.0054 ± 0.00073 | | |
| *ATM* | BM | 8 | 0.0021 ± 0.00414 | 2.03 | 0.430 |
| | PB | 15 | 0.0009 ± 0.00034 | | |
| *ATR* | BM | 8 | 0.0016 ± 0.00342 | 1.13 | 0.309 |
| | PB | 15 | 0.0003 ± 0.00008 | | |
| *CASP9* | BM | 8 | 0.0026 ± 0.00513 | 1.46 | 0.432 |
| | PB | 15 | 0.0011 ± 0.00016 | | |
| *CDK4* | BM | 8 | 0.0036 ± 0.00623 | 0.68 | 0.249 |
| | PB | 15 | 0.0009 ± 0.00021 | | |
| *CDKN1A* | BM | 8 | 0.0021 ± 0.00273 | 2.47 | 0.850 |
| | PB | 15 | 0.0019 ± 0.00060 | | |
| *CHEK2* | BM | 8 | 0.0031 ± 0.00763 | 0.40 | 0.316 |
| | PB | 15 | 0.0002 ± 0.00005 | | |
| *E2F1* | BM | 8 | 0.0007 ± 0.00081 | 0.46 | 0.066 |
| | PB | 15 | 0.0001 ± 0.00006 | | |
| *E2F3* | BM | 8 | 0.0021 ± 0.00126 | 0.99 | 0.286 |
| | PB | 15 | 0.0016 ± 0.00023 | | |
| *MCL1* | BM | 8 | 0.0781 ± 0.05242 | 0.98 | 0.350 |
| | PB | 15 | 0.0593 ± 0.01336 | | |
| *MDM2* | BM | 8 | 0.0165 ± 0.03737 | 1.09 | 0.393 |
| | PB | 15 | 0.0045 ± 0.00103 | | |
| *MDM4* | BM | 8 | 0.0013 ± 0.00183 | 1.31 | 0.555 |
| | PB | 15 | 0.0009 ± 0.00031 | | |
| *PTEN* | BM | 8 | 0.0196 ± 0.01991 | 0.91 | 0.362 |
| | PB | 15 | 0.0127 ± 0.00146 | | |
| *RB1* | BM | 8 | 0.0281 ± 0.07244 | 0.42 | 0.336 |
| | PB | 15 | 0.0016 ± 0.00036 | | |
| *P53* | BM | 8 | 0.0482 ± 0.12947 | 0.64 | 0.353 |
| | PB | 15 | 0.0027 ± 0.00052 | | |
| *BCL2* | BM | 8 | 0.3217 ± 0.90720 | 0.38 | 0.351 |
| | PB | 15 | 0.0009 ± 0.00035 | | |
| *CHEK1* | BM | 8 | 0.0091 ± 0.02359 | 0.09 | 0.315 |
| | PB | 15 | 0.0001 ± 0.00002 | | |
| *GADD45* | BM | 8 | 0.0011 ± 0.00063 | 0.10 | **0.003** |
| | PB | 15 | 0.0001 ± 0.00002 | | |
| *PCNA* | BM | 8 | 0.0263 ± 0.04768 | 0.23 | 0.202 |
| | PB | 15 | 0.0026 ± 0.00059 | | |
| *PTX3* | BM | 8 | 0.0035 ± 0.00357 | 0.06 | **0.033** |
| | PB | 15 | 0.0002 ± 0.00009 | | |

PB: Peripheral blood; BM: Bone marrow

BM extraction (6). BM aspiration from these regions is highly invasive and local anesthesia is applied during the procedure. It has been reported that these anesthetics may have side effects that lead to various neurodevelopmental disorders, particularly in children (7). Allogeneic hematopoietic stem cell transplantation in adults with hematological malignancies is increasingly performed using PB-derived stem cells rather than BM. It is possible to donate PB-derived stem cells for convenience and safety reasons as well as logistical reasons, which have been behind the growth of this stem cell source (8).

Gene expression analyses in samples from PB mononuclear cells and different tissues are not always the same. Results from a single tissue are not sufficient to explain how expression changes affect complex biological systems in human beings (9). Limited studies compare PB and BM samples in terms of expression patterns. As a result of our detailed investigation, no study was present in the literature comparing the *p53* pathway gene expression levels in healthy individuals. *HMG-CoA reductase gene (HMGCR)* and *low-density lipoprotein receptor (LDLR)* gene expressions in the PB and BM samples of myelodysplastic syndrome (MDS) patients have been reported to be upregulated similarly when compared to blood samples of healthy controls (10). Apoptosis stimulating effect of p53 protein 2 (*ASPP2)* gene expression in PB and BM samples from chronic lymphoblastic leukemia (CLL) patients has been reported to be downregulated in the same way when compared to blood samples from healthy controls. In the same study, it was shown that the level of gene expression in the PB and BM samples of the patients were similar (11).

The p53 pathway genes, mainly p53, respond to stress during cell division (12). However, tumor protein p53 (TP53) is the most frequently genetically altered gene in both solid and hematological cancers (13). Other genes involved in this pathway have not been elucidated as p53. In order to understand the similar and different aspects of PB and BM aspirate materials in terms of p53 pathway genes, we aimed to compare the differential expression patterns of 22 genes ATM serine/threonine kinase *(ATM)*, Bcl-2 associated X-protein (*BAX*), cyclin-dependent kinase 4 *(CDK4)*, cyclin dependent kinase inhibitor 2A *(CDKN2A),* checkpoint kinase 1 *(CHEK1),* checkpoint kinase 2 *(CHEK2),* cyclin dependent kinase inhibitor 1A *(CDKN1A),* Ataxia Telangiectasia and Rad3 related *(ATR),* growth arrest and DNA damage inducible alpha *(GADD45A),* mouse double minute 2 homolog *(MDM2),* mouse double minute 4 *(MDM4),* proliferating cell nuclear antigen *(PCNA),* retinoblastoma 1 *(RB1),* caspase 9 *(CASP9),* tumor protein P53 *(TP53)*, B-cell lymphoma 2 *(BCL2),* apoptotic peptidase activating factor 1 *(APAF1),* E2F transcription factor 1 *(E2F1),* E2F transcription factor 3 *(E2F3),* myeloid cell leukemia 1 *(MCL1),* pentraxin 3 *(PTX3),* phosphatase and tensin homolog *(PTEN)* selected from the p53 pathway involved in cell growth, proliferation, differentiation, DNA repair and apoptosis processes.

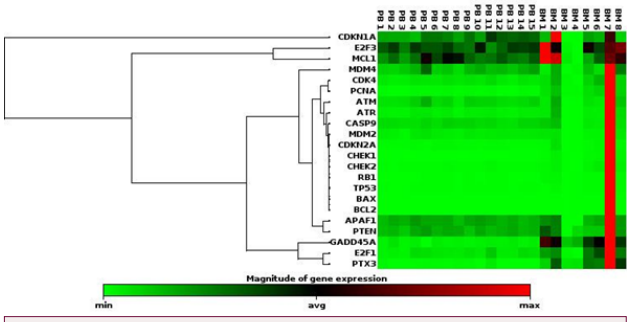


**Figure 1.** The average of the fold change for PB and BM samples were shown in heatmap graphs. The heat map is a graphical representation of the $RT^2$-PCR array's findings about the differential regulation of gene expression between the peripheral blood and bone marrow samples. The intensity of the colors reveals the degree of change in gene expression. Low gene expression (ratio<1) in PB samples is shown in green. Genes with a ratio close to 1 are shown as black squares. Gene expression in the red squares is greater than in the BM samples (ratio>1).

## MATERIALS AND METHODS

### Samples

PB and BM samples were collected in sterile tubes containing EDTA, after obtaining written informed consent from 23 healthy volunteers. PB samples were taken from healthy hospital personnel without a history of leukemia, and BM samples were taken from BM transplantation donor candidates in the Istanbul University Faculty of Medicine, Department of Hematology. The median age of 50.95 years was observed among 10 males (43.4%) and 13 females (56.5%). The study protocol was designed in accordance with the Declaration of

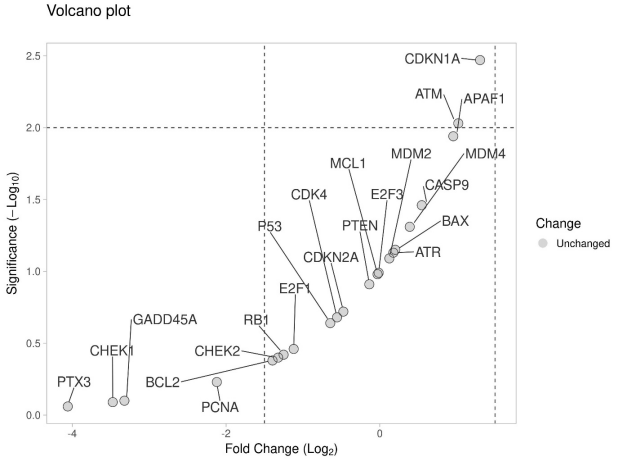


**Figure 2.** A volcano plot was generated showing the gene expressions of BM samples versus PB samples. Distributions of fold changes in gene expression are shown using volcano plots. BM samples compared with PM samples. p-values less than 0.05 and absolute fold changes of 2 were considered significant. No significant variation in fold alterations of genes was observed.

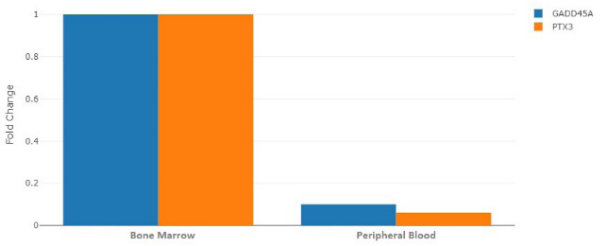


**Figure 3.** Comparison of the *GADD45* and *PTX3* gene expression in PB and BM samples. Fold-change ($2^{-\Delta\Delta CT}$) is the ratio of the normalized gene expression ($2^{-\Delta CT}$) in the PB sample to the normalized gene expression ($2^{-\Delta CT}$) in the BM sample. Fold-change values higher than one indicate a positive regulation, also known as an up-regulation, whereas fold-change values lower than one indicate a negative regulation, also known as a down-regulation.

Helsinki. The study was conducted with the ethical approval obtained from the Clinical Research Ethics Committee of Istanbul University Faculty of Medicine (E-29624016-050.99-876968/ May 9, 2022).

## RNA Isolation and cDNA Synthesis

Total RNA from whole blood and BM samples were extracted using the QiaAmp RNA Blood Mini Kit (Qiagen, USA). RNA quality and quantity were measured using NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). A template of 1 µg of total RNA was used in cDNA synthesis. cDNA synthesis was performed on a LightCycler 480 II platform using Qiagen RT²HT First Strand Kit (Qiagen, USA).

## Expression Analysis via qRT-PCR Array

qRT-PCR array using a custom RT² - Profiler PCR array (CAPH_133446F; cat no. 330131/12 plate) was conducted to analyze the mRNA expressions of 22 genes involved in proliferation, differentiation, cell cycle, DNA repair, and apoptosis processes intervened by the p53 pathway. The study was carried out on a LightCycler 480 II platform using SYBR Green master mix and 1 µl cDNA sample. The gene expression levels were analyzed and normalized with the β-actin housekeeping gene. The Livak method was used to calculate relative gene expression levels (14).

## Statistical Analysis

Statistical analysis was carried out using the SPSS package program, version 21. Statistical analysis of the expression levels of the genes in the BM aspirate and PB was carried out using the Student's t-test. Pearson correlation analysis was conducted to determine the expression correlations of genes that were statistically significant between the groups. $p<0.05$ was determined as the cut-off value for statistical significance.

## RESULTS

Two of the p53 pathway genes *(GADD45* and *PTX3*) we investigated in our study were found to have different expression levels between BM and PB. The fold change in expression of these two genes between the groups (BM/PB) was 0.1 and 0.06, respectively. It was found that *GADD45A* and *PTX3* gene expressions were significantly decreased in BM samples compared to PB samples ($p=0.003$ and $p=0.033$, respectively) (Table 1). However, there was no statistical difference in the expression levels of *APAF1, ATM, BAX, CDKN1A, CDKN2A, MDM2, MCL1, MDM4, PTEN, CASP9, ATR, CHEK2, E2F1, E2F3, RB1, TP53, BCL2, CDK4, CHEK1,* and *PCNA* genes between the two groups (Table 1). The averages of the fold change for PB and BM samples were shown in heatmap graphs (Figure 1). A volcano plot was generated showing the gene expressions of BM samples versus PB samples (Figure 2). Comparison of the gene expressions of the genes that are differentially expressed (*GADD45, PTX3*) are shown in the bar graph (Figure 3).

According to the results of the Pearson correlation analysis, it was observed that the *GADD45* gene showed a high correlation with the expressions of the *APAF1, ATR, CDK4, E2F1, PTEN, PCNA, PTX3* genes ($r=0.7$; $p<0.001$). In addition, the *PTX3* gene showed a high correlation with the expressions of *BAX, CDKN2A, APAF1, ATM, ATR, CASP9, CDK4, CHECK2, E2F1, MDM2, MDM4, PTEN, RB1, TP53, BCL2, CHECK1, GADD45* and *PCNA* genes ($r=0.7$; $p<0.001$). The correlation between *GADD45* and *PTX3* was quite high ($r=0.886$, $p<0.0001$).

## DISCUSSION

Transcription factor p53, which is one of the major tumor suppressor genes, affects the expression of many genes by targeting more than thousands of sites (15). p53 plays a role in the regulation of many cellular metabolic functions. In addition, p53 is also involved in various processes, including the regulation of growth factors and reactive oxygen species, which have an impact on cancer pathogenesis (16). Disturbances in the p53 pathway play a more active role in some hematological cancers like chronic lymphoblastic leukemia (CLL) and multiple myeloma (17, 18). Hematological cancers are malignancies of BM-derived cells. BM aspiration biopsy, which is important in the diagnosis and routine follow-up of patients, is an invasive technique that is very uncomfortable for the patient and has risks compared to PB collection. It is very important to determine the expression patterns of p53 pathway genes in PB and BM, as they may contribute to the same mechanisms. To the best of our knowledge, our study is the first to compare the differences in mRNA levels and correlations of p53 pathway genes between PB and BM samples.

However, there are various studies in the literature investigating different gene expression states in PB and BM samples of patients with hematological cancers. Crassini et al. examined the expression profile of 260 genes in PB, BM, and lymph node-derived CLL cells. They reported that approximately half of these

genes were reduced in PB-induced CLL cells compared to other groups (19). In another study comparing the expression levels of PB and BM samples taken from CLL patients, it was stated that there was not much difference in *Bcl-2, ADAM29, Mcl-1*, *ZAP70,* and *LPL* genes in this sense (20). In their study on acute myeloid leukemia patients, Sakhinia et al. did not find any difference in the expression of some genes *(leptin receptor, CD33, adipsin, proteoglycan 1, MB- 1, cyclin D3, hSNF2b, proteasome iota, HkrT-1,* and *E2A),* but they found significant changes in the expression of some other genes *c-myb, HOXA9, LYN, cystatin c* and *LTC4s* (21). In a study conducted on patients with chronic myeloid leukemia, it was reported that BCR-ABL transcript levels were correlated in PB and BM samples, and PB could be used instead of BM in routine monitoring (22). Moreover, van Leeuwen-Kerkhoff et al. reported that *MRC1, CSF3R, XCR1, CLEC9A* and *IRF8KI* gene expressions were correlative in BM and PB-derived myeloid dendritic cell subgroups (23).

Low fold change value detected in *GADD45* and *PTX3* genes and the presence of insignificant gene expression levels in other p53 pathway genes may indicate the similarity of PB and BM with respect to p53 pathway gene expressions.

Members of the GADD45 family, which are defined as stress sensors, are triggered by environmental stress such as inflammatory cytokines and genotoxic agents (24, 25). They also regulate genomic stability, senescence, cell survival and apoptosis (24). Apoptosis is induced when DNA damage is fatal or GADD45A arrests cell cycle progression. The hematopoietic system might benefit from GADD45A's ability to induce terminal differentiation in damaged stem and progenitor cells, as well as DNA repair and genomic stabilization (26). The impact of GADD45 proteins on tumor growth depends on the molecular structure of the activated oncogene, as well as the cell type in which it is expressed and the signaling pathways with which it is currently interacting (25). Exemplarily, GADD45a is both expressed independently of p53 and is a p53 target gene (27).

The second gene, *PTX3* which is a multifunctional protein, plays an important role in immunity, inflammation, and extracellular matrix organization/remodeling (28). In studies investigating the role of *PTX*3, it has been reported that it is expressed in different cells such as macrophages, neutrophils, and smooth muscle cells (29). According to the data we obtained from our study, a strong correlation was identified between *GADD45* and *PTX3* gene expression levels. Because of the similar mRNA levels between PB and BM, we think that PB may reflect the BM expression pattern for the other p53 pathway genes we analyzed.

As a limitation of our current study, the number of the participants was relatively small, that's why it should be considered as a preliminary study and should be confirmed by further studies encompassing larger PB and BM groups.

In this present study that aimed to compare the expression levels of the important genes in the p53 pathway, we detected a statistically significant difference between the expression patterns of only two genes (*GADD45A, PTX3)* in the PB and BM out of 22 genes. The fact that no statistically significant difference was observed in the expression of the other 20 genes in both groups suggested that these two groups may have similar characteristics in terms of *p53* pathway genes. Therefore, a PB sample, which is easier and less invasive to obtain, may be a convenient alternative to examine the expressions of genes in the p53 pathway instead of the BM sample.

**Ethics Committee Approval:** This study was approved by Clinical Research Ethic Committee of Istanbul Faculty of Medicine (E-29624016-050.99-876968/ May 9, 2022).

**Authors' Contributions:** Conception/Design of Study – A.D.A., G.O., I.S.; Data Acquisition – A.D.A., G.O., I.S.; Data Analysis/Interpretation – A.D.A., G.O., I.S.; Drafting Manuscript– A.D.A., G.O., I.S.; Critical Revision of Manuscript- A.D.A., G.O., I.S.; Final Approval and Accountability– A.D.A., G.O., I.S.

**Note:** All authors have contributed equally to this work.

**Conflict of Interest:** Authors declared no conflict of interest.

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

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Experimed 2023; 13(2): 109-114

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1301807

# Investigation of the Toxicologic and Biochemical Effects of Silk Fibroin/Gold Nanoparticles-Based Nanofiber Using Zebrafish Embryos

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**Cite this article as:** Ozcan O, Unal I, Tufan E, Emekli-Alturfan E, Tunali-Akbay T. Investigation of the toxicologic and biochemical effects of silk fibroin/gold nanoparticles-based nanofiber using zebrafish embryos. Experimed 2023; 13(2): 109-114.

## ABSTRACT

**Objective:** This study aimed to test the toxicity of silk fibroin (SF) / gold nanoparticles (AuNPs)-based nanofiber by using zebrafish embryos as an alternative animal model.

**Materials and Methods:** Nanofiber was fabricated via electrospinning. The zebrafish embryos were divided into four groups as control, 3,4-dichloroaniline (DCA) treated, one day SF/AuNPs treated (1D), and seven days SF/AuNPs treated (7D) group. The SF/AuNPs nanofiber was incubated in the medium for one day and seven days. Following incubation, the embryos were placed in the mediums and their development was monitored 72 hours post-fertilization. In the zebrafish embryos, levels of malondialdehyde (MDA), nitric oxide (NO), activities of superoxide dismutase (SOD) and glutathione-S-transferase (GST) were detected.

**Results:** Compared to the control group, there was no change in the hatching and mortality rates in the 1D and 7D groups. In the DCA group, the mortality rate was higher than the controls. In the 1D and 7D groups, MDA and NO were higher than the control but lower than the DCA group. SOD and GST activities decreased compared to the control.

**Conclusion:** SF/AuNPs-based nanofiber did not affect the hatchability and mortality of embryos but increased oxidant damage, therefore it is thought that this oxidant effect of SF/AuNPs-based nanofibers may provide antibacterial properties.

**Keywords:** Silk fibrion, gold nanoparticles, zebrafish embryos, oxidant-antioxidant status, toxicity

## INTRODUCTION

The development of functional nanofiber-based membranes has gained considerable interest due to their distinctive features like high level porosity, high surface area-to-volume ratio, and excellent biocompatibility (1). In this study, gold nanoparticles (AuNPs)-added silk fibroin (SF)-based nanofiber membranes were prepared. Among various materials studied for nanofibrous membranes, SF has attracted interest due to its biodegradability and low immunogenicity. SF is a natural protein produced by a variety of spiders and insect species that has remarkable strength, toughness, and flexibility (2). Human epithelial, fibroblast, keratinocyte, and osteoblast cells from a variety of tissues may adhere to, spread out, and develop when placed in three-dimensional SF nets. (3). SF has been utilized in bioengineering delivery systems for drugs, and delivery of therapeutics to cancer cells (4). SF has high biocompatibility, controlled degradability, structural integrity, and diverse process ability, these features make SF a desirable and valuable candidate for use in tissue science and delivery systems for drugs (5). Furthermore, SF nanofibers have been found to exhibit antioxidant effects, which can further support the wound-healing process

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**Submitted:** 24.05.2023 **Revision Requested:** 16.06.2023 **Last Revision Received:** 16.06.2023 **Accepted:** 21.06.2023 **Published Online:** 26.07.2023

(6). The addition of AuNPs to SF may exhibit bioactivities that could be used in biomedical applications. From a biological standpoint, the toxicity of AuNPs is crucial (7). In this case, the preparation of AuNPs, the dose and toxicity of AuNPs in products such as wound dressings in which nanoparticles are used needs to be considered. Following investigation of AuNPs in fibroblasts, epithelial cells, and macrophages, there was substantial concern that small AuNPs (dcore 2.0nm), which are more redox-reactive than bigger AuNPs, would have significant toxicity. However, further *in vitro* and *in vivo* studies have revealed that AuNPs, regardless of core diameter, are not acutely hazardous or toxic (8).

However, some studies state the AuNPs-induced reactive oxygen species production causes toxic effects on the DNA, cell nucleus, and mitochondria (9). Researchers have difficulty due to a lack of understanding of the mechanisms involved in the reduction stability and effects of biosynthesized gold nanoparticles (10). In this study gold nanoparticles were synthesized by using green chemistry techniques to decrease their hazardous effects and mixed with the SF to fabricate SF/AuNPs hybrid electrospun nanofiber.

The production of hybrid nanofibers containing gold nanoparticles are an important option in the invention of clean, nontoxic, and sustainable products with significant benefits over previous methods (11). Therefore, in this study, the possible toxicity of SF/AuNPs -based nanofibers was tested using zebrafish embryos focusing on the oxidant-antioxidant system when used as a wound dressing material.

## MATERIALS AND METHODS

### SF Preparation

SF was synthesized according to Zhu et al. (12). To degum the silk from sericin, Bombyx mori silk cocoons (5g) were cut into portions and boiled for thirty minutes in a 0.02 M sodium carbonate solution. The degummed silk was washed with deionized water and dried before being dissolved for four hours in 9.3 M LiBr solution and then dialyzed against deionized water for 72 hours to remove the LiBr. The obtained SF was centrifuged at 9,500xg and kept in 4°C.

### AuNPs Preparation

AuNPs were prepared by using green chemistry. Collected fresh strawberry leaves were dried in a cool and shaded area. The dried strawberry leaves were then pulverized in a mortar. To ensure an equal size of the particles, the powder was sieved with a pore size of approximately 0.08mm sieve. 0.4 grams of strawberry leaf powder was mixed with deionized water and heated at 80°C for 20 minutes to create an aqueous extract. The plant extract was cooled to room temperature, and a simple centrifugation process was employed to remove the plant debris. A 1mM concentration of $HAuCl_4$ (chloroauric acid) solution was used to obtain gold nanoparticles. The 1mM $HAuCl_4$ solution was heated to 100$^0$C. The strawberry leaf extract was added to the boiling gold solution. Heating was continued until the prepared solution reached a dark red color. After the color change in approximately one to five seconds, the prepared solution was cooled to room temperature (13).

### Preparation of SF /AuNP Nanofiber

10% SF in formic acid was mixed with 1% gold nanoparticles. This hybrid mixture was used to produce nanofiber. The optimized conditions for the electrospinning were determined as 25.5 kV electrical power, the flow rate in the feeding unit was 0.15mL per minute, the collector rotation speed was 200 rpm and the distance between the nozzle and the collector was 13.7 cm.

### Development of Zebrafish Embryos

The zebrafish (AB/AB strain, wild type) was kept in an environment which was free of disease under controlled care. The zebrafish were maintained in an aquarium racking mechanism (ZebTEC, Italy) between 26-28$^0$C. They were fed with commercial powdered fish meal twice a day, mixed with live saltwater shrimp. All studies were conducted in 0.018 mg/L sea salt solution which was prepared with reverse osmosis water (Instant OceanTM, USA). For all experiments, reverse osmosis water supplemented with 0.018 mg/L sea salt (Instant OceanTM, USA) was used. After natural breeding, fertilized embryos were obtained, cultured, and classified based on development time and characteristic morphology using a Zeiss Discovery V8 stereomicroscope (Germany) (14).

### Experimental Design of Zebrafish Embryo Groups

Zebrafish embryos were divided into four groups as control, 3,4-Dichloroaniline (DCA, Fluka, Steinheim, Germany), one-day (1D) and seven-day incubation (7D) groups. No treatment was applied to the embryo medium of the control group. In the DCA group, 4mg DCA/L was added to the embryo medium. In the 1D incubation group, 3x3cm SF/AuNPs based nanofiber was, continuously shaken in the embryo medium for 24 hours, and in the 7D incubation group 3x3 cm SF/AuNPs based nanofiber was continuously shaken in the embryo medium for seven days. Embryos were exposed to four different embryo mediums and monitored for 72 hours post-fertilization (hpf).

### Biochemical Analysis

### Preparation of Zebrafish Embryos for Biochemical Analysis

Biochemical analyses were conducted on the zebrafish embryos. The embryos were produced as five biological replicates of zebrafish embryos (100 embryos/pool) through the use of 72 hours post-fertilization (hpf) zebrafish. One milliliter phosphate-buffered saline (PBS) was used to homogenize 100 embryos in each pool and then the pools were centrifuged. Biochemical analysis was performed using the supernatant.

## Lipid Peroxidation and Nitric Oxide (NO) Levels

The Ledwozyw method was used to measure the level of malondialdehyde (MDA), which serves as a lipid peroxidation end product, using thiobarbituric acid reactive substances (15). The MDA level was presented as nmol MDA/mL.

The NO measurement was conducted using the Miranda et al. method (16). In the NO determination, vanadium (III) chloride was used to reduce nitrate to nitrite. Under acidic conditions, the nitrite produced reacts with sulfonyl amide and N-(1-naphtyl) ethylenediamine dihydrochloride, forming a complex diazonium compound. The intensity of the resulting complex was measured at 540nm. The results were presented as µmol NO/mL.

## Superoxide Dismutase (SOD) and Glutathione-S-Transferase (GST) Activities

The SOD activity was measured with a method that consist on the potential of SOD to enhance the effects of riboflavin-sensitized photooxidation of o-dianisidine. The resulting product's absorbance was determined at 460nm. The net absorbance was calculated by comparing the absorbance values at zero and eight minutes of illumination. The SOD activity was presented as U/mL (17).

The GST activity was measured using the Habig et al. method. The absorbance of the formed product was detected at 340nm wavelength using a spectrophotometer (18).

## Statistical Analysis

One-way analysis of the variance (ANOVA) and post hoc Tukey's multiple comparison tests were used to analyze the differences between the groups using the Graph Pad 8 (GraphPad Software, La Jolla, CA, USA). $p<0.05$ was considered significant.

# RESULTS

## Development, Mortality and Hatching Rates of the Embryos

The mortality rates of zebrafish embryos at 72 hpf were presented in Figure 1. The mortality rate was significantly increased in embryos exposed to DCA when compared to the control group. Mortality rates in the 1D and 7D exposure groups were not significantly changed when compared to the control group. The hatching rates of zebrafish embryos at 72 hpf are presented in Figure 2. There was no significance between the hatching rates of all groups.

## Lipid Peroxidation and NO Levels

The MDA and NO levels significantly increased in all groups when compared to the control group (Figure 3). The MDA and NO levels of the 1D and 7D groups were significantly lower than the DCA group (Figure 3).

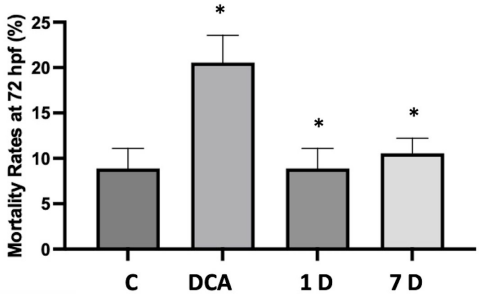


**Figure 1.** The mortality rates of zebrafish embryos exposed to DCA and also SF/AuNPs for 1 day (1D) and 7 days (7D) were measured at 72 hours post-fertilization (hpf).
C:Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7.
*p< 0.05 significantly different from the control group (C).

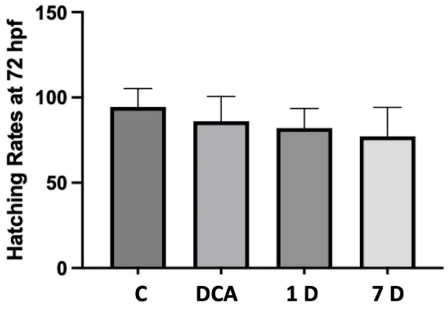


**Figure 2.** The hatching rates of zebrafish embryos exposed to DCA and also SF/AuNPs for 1 day (1D) and 7 days (7D) were measured at 72 hours post-fertilization (hpf).
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7.

## SOD and GST Activities

The SOD activity in all groups was lower than the control group (Figure 4). The GST activity was significantly decreased in the DCA and 1D groups when compared to the control group, while the 7D group showed similar GST activity to the control group (Figure 4).

## Discussion

In health-related products, gold nanoparticles and SF-based products continue to generate great attention. However, since there are dilemmas related to products containing these substances in the studies, assessing the potential toxicity

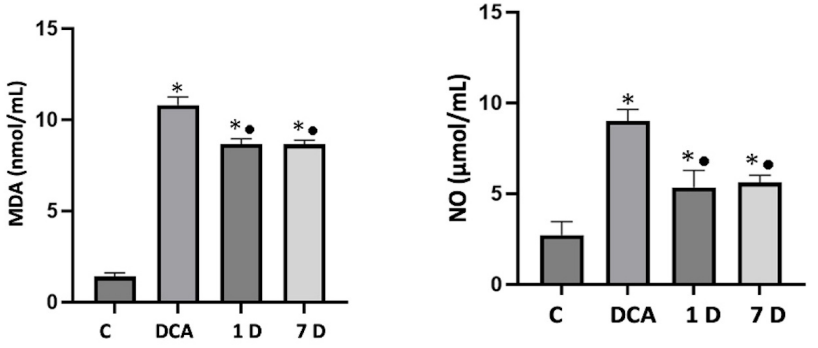


**Figure 3.** Malondialdehyde and nitric oxide levels of zebrafish embryos at 72 hpf.
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7,* $p < 0.05$ significantly different from the control group, • $p < 0.05$ significantly different from the DCA group

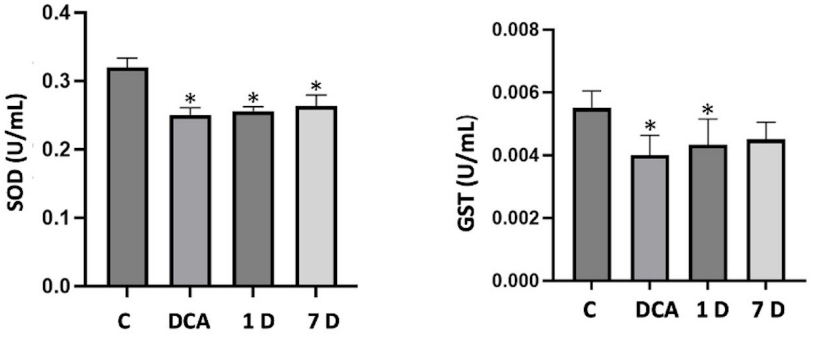


**Figure 4.** Superoxide dismutase and glutathione-S-transferase activities of zebrafish embryos at 72 hpf.
C: Control
DCA: 3,4-Dichloroaniline
1D: Silk fibroin/gold nanoparticles treatment at day 1
7D: Silk fibroin/gold nanoparticles treatment at day 7 * $p < 0.05$ significantly different from the control group.

of the SF/AuNPs-based hybrid nanofiber was necessary. Therefore, this study demonstrated the possible toxic and oxidant-antioxidant effects of SF/AuNPs-based nanofibrous membranes. According to the results of this study, the SF/AuNPs-based nanofibers had a nontoxic effect on zebrafish embryos and promoted the healthy development of zebrafish embryos compared to the DCA exposed zebrafish embryos as positive control. Higher hatching rates and lower mortality rates were observed in groups treated with SF/AuNPs -based nanofiber when compared to the DCA-exposed zebrafish embryos. It was revealed that gold nanoparticles do not exhibit any toxicity to zebrafish embryos when compared to other nanoparticles like silver, copper, and platinum (19). However, there is no research on how gold nanoparticles affect the zebrafish embryos when they form a hybrid with SF. The toxicity of SF/AuNPs hybrid nanofibers depend on several factors, including the characteristics of the nanoparticles, their concentration, exposure duration, and the specific biological system under consideration. While SF is generally considered biocompatible, the addition of gold nanoparticles could potentially introduce new properties and interactions that may affect the toxicity (20).

Exposure to SF/AuNPs-based nanofiber increased lipid peroxidation in the embryos. Under certain conditions, gold

nanoparticles may release and could contribute to oxidative damage. They could induce the generation of reactive oxygen. Excessive production of reactive oxygen species production disrupts the oxidant-antioxidant balance and leads to oxidative stress. The release of gold ions depends on factors such as pH, temperature, and exposure duration (21, 22). In this study, the increased lipid peroxidation level and decreased SOD and GST activities in the zebrafish embryos could be related to the gold nanoparticles released from the hybrid nanofiber. NO is another reactive oxygen species, and the current study found that embryonic NO levels increased in response to DCA and nanofibrous membranes (23). NO is a highly reactive physiological molecule that has been revealed to play a vital function in controlling pre-implantation embryo development (24). Gouge et al. revealed the need for NO in embryos before implantation and suggests that it may play a function in controlling mitosis in them (25). The present findings show that DCA and nanofibrous membrane resulted in higher NO generation in 72 hpf embryos when compared to the control group but this increase in the NO level did not affect the hatching and mortality rate of the zebrafish embryos. The oxidant effect of the SF/AuNPs nanofiber membrane exposed embryo medium showed that gold nanoparticles were released into the medium. This oxidant effect of the membrane could contribute to killing the bacteria by adhering to their surface and could cause oxidant damage to bacteria when used as a wound-healing material. This confirmed that the membrane produced in this study could have antibacterial properties. The dimension, shape, charge, and coating of the surface of gold nanoparticles released to the embryo medium could also influence their oxidant-antioxidant balance (26). The size of the gold nanoparticles that were used in this study was approximately 20 nm. Smaller nanoparticles, especially those below ten nanometers, have greater potential for cellular uptake and interaction and could alter the behavior of gold nanoparticles (27).

In conclusion, while SF is considered biocompatible, the addition of gold nanoparticles introduces a new component that could have an oxidant and therefore antibacterial effect on the hybrid nanofibers. Therefore, a careful evaluation of the specific hybrid nanofiber formulation is necessary to assess its safety and potential toxicity in the intended application.

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**Ethics Committee Approval:** According to the legal regulations in the European Union and in our country, ethical committee approval is not required for the use of embryos and larvae in studies involving zebrafish during the first 120 hours of post-fertilization development. In this study, the research was terminated at 72 hours post-fertilization.

**Authors' Contributions:** Conception/Design of Study – O.O., T.T.A., E.E.A.; Data Acquisition – E.E.A., I.U., O.O., E.T.; Data Analysis/Interpretation – T.T.A., E.E.A.; Drafting Manuscript– T.T.A., O.O; Final Approval and Accountability– T.T.A., E.E.A.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Financial Disclosure:** This study was funded by a grant from Marmara University Scientific Research Project Department (Project ID: TDK-2022-10384).

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Experimed 2023; 13(2): 115-120

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1301967

# *In vitro* Antileishmanial Activity of *Lavandula angustifolia* Essential Oil on *Leishmania infantum* Parasites

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**Cite this article as:** Islek Z, Sahin F. *In vitro* antileishmanial activity of *Lavandula angustifolia* essential oil on *Leishmania infantum* parasites. Experimed 2023; 13(2): 115-120.

## ABSTRACT

**Objective:** Leishmaniasis is an endemic tropical disease that is disseminated through the bite of a sandfly infected with *Leishmania* parasites. Conventional antileishmanial drugs are mainly toxic and can be ineffective; therefore, there is a need for new natural drug candidates. This study investigated the antileishmanial effects of *Lavandula angustifolia* (LA) essential oil on *Leishmania infantum (L. infantum)* parasites, and the safety features were tested on RAW264.7 murine macrophages.

**Materials and Methods:** LA essential oil was produced through the process of hydro-distillation, and its phytochemical content was determined using the gas chromatography-mass spectrometry (GC-MS) analysis. The antileishmanial effects of LA (0.063 to1 µL/mL) on *L. infantum* parasites as well as their safety features were assessed on RAW264.7 murine macrophages.

**Results:** The composition of LA essential oil was detected using GC-MS analysis, including linalool, pinene, 1, 8-cineole, linalyl acetate, and lavandulol. Concentrations at and above 0.5 µL/mL LA indicated a significant reduction (71% decrease) in the parasite proliferation, and caused a slight reduction in macrophage viability to 70% at 72 h.

**Conclusions:** The findings revealed the antileishmanial effect of LA on *L. infantum* parasites with relatively less toxicity on macrophages. The promising antileishmanial efficacy highlights the potential for further *in vivo* studies.

**Keywords:** Antileishmanial therapy, *Lavandula angustifolia*, *Leishmania infantum*, macrophages

## INTRODUCTION

Leishmaniasis is a parasitic disease widespread in tropical regions, caused by protozoan parasites classified under the genus *Leishmania*. It significantly affects millions of individuals globally, particularly in developing countries, and is transmitted via the bite of phlebotomine sandflies. More than 20 different *Leishmania* species exhibit the ability to survive as either promastigotes (extracellular form) within sandflies or amastigotes (intracellular form) inside mammalian macrophages (1). According to a report by the World Health Organization in 2018, leishmaniasis is widespread in 98 tropical countries, thereby posing a significant threat to more than 600,000 individuals (2). There is an annual occurrence of around 1.3 million new cases of leishmaniasis, which manifest in three clinically different forms, including visceral Leishmaniasis, which is generally known as "Kala Azar" and can lead to mortality, if not treated.

The current treatment options available for Leishmaniasis have been reported to contain pentavalent antimonials, conventional amphotericin B (AmpB) deoxycholate, miltefosine, paromomycin, AmpB micelles (Fungizone), and liposomal AmpB (AmBisome) (3–9). However, therapeutic interventions have certain limitations associated with these therapies, such as restricted efficacy against parasites (10), numerous adverse effects owing to low therapeutic

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**Submitted:** 24.05.2023 **Revision Requested:** 14.06.2023 **Last Revision Received:** 14.06.2023 **Accepted:** 23.06.2023 **Published Online:** 04.08.2023

index (11), the need for cautious and gradual intravenous administration (e.g. AmpB) (12), and the emergence of drug resistance against the therapy (3, 5, 7, 13– 17). Therefore, there is a need for new drugs with fewer side effects and higher efficacy.

In recent years, researchers have turned their attention to natural products as potential candidates for new drugs (18–24). The plant Lavandula, commonly referred to as lavender, has been utilized for its medicinal properties for centuries. Previous studies demonstrated that lavender exhibits diverse biological activities, including anti-protozoal effects against Leishmaniasis and other protozoan infections (19, 25, 26). Besides, the essential oils of *Lavandula angustifolia* (LA) and *Lavandula intermedia* were assessed for their anti-parasitic properties against protozoans, namely *Giardia duodenalis, and Trichomonas vaginalis*, as reported in a previous study (26). In another study, the antileishmanial effects of LA and *Rosmarinus (R.) officinalis* essential oils, as well as their nanoemulsions against *Leishmania major*, were examined by Shokri et al. (2017) (19). Upon treatment of *Leishmania* parasites with different species of lavender, several compounds were investigated in the composition of lavender, such as linalool and linalyl acetate, which displayed a potent antileishmanial activity. This has led to increased interest in exploring lavender as a potential source of new drugs for leishmaniasis.

In this study, LA essential oil (found in Turkish flora) was first obtained by hydro-distillation, and further composition of LA was detected by gas chromatography-mass spectrometry (GC-MS) analysis. Following the investigation of the plant composition, the antileishmanial effect of LA on the growth of *Leishmania infantum (L. infantum)* parasites was evaluated together with its safety properties on healthy murine RAW264.7 macrophages at different ranges of concentrations (0.063-1 µL/mL). Cell culture studies, including cell viability, and morphology were conducted to evaluate the antileishmanial efficacy of LA essential oil.

## MATERIALS AND METHODS

### Materials

Resazurin sodium salt (R7017 - 1G) was purchased from Sigma-Aldrich, Germany. The flowering aerial tops of LA were collected from Yalova, Turkiye, in May 2022. The plant material was identified by Prof. Dr. Fikrettin Sahin.

### The Process of Obtaining LA Essential Oil Through Hydro-Distillation

A quantity of 200 grams of herbal material that had been dried and crushed was subjected to distillation with 2 liters of pure water for a duration of 3 h, utilizing a Clevenger-type apparatus.

### GC-MS Analysis

The carrier gas in the experiment was helium, which was maintained at a consistent flow rate (1 mL/min). 1 µL sample was introduced into the system via injection. The temperature program for the GC was established in the following manner: a hold at 50°C for 5 minutes, subsequently a ramp to 250°C at a rate of 5°C /minute, and a subsequent hold for 10 minutes. The transfer line for the mass spectrometer was maintained at 220°C. The mass range of 50 to 650 m/z was used in scan mode, and as a column, DB-Wax 60 m x 0.25mm ID x 0.25 µm was utilized.

### Parasite Culture

The *L. infantum* (MHOM/MA/67/ITMAP-263) strain was obtained from Dr. Ana M. Tomás at the University of Porto, Portugal. The cultivation of *L. infantum* promastigotes was performed in RPMI 1640 Glutamax medium, which was supplemented with 10% (v/v) inactivated fetal bovine serum (iFBS), 50 U/mL penicillin, 50 µg/mL streptomycin, and 20 mM HEPES sodium salt at 25°C. The collection of promastigotes was carried out through centrifugation of the pellet at 3000 × g for 10 minutes.

### Macrophage Culture

The RAW 264.7 murine macrophage cells (American Type Culture Collection TIB-71) were cultured in DMEM that was supplemented with 10% iFBS, 2 mM L-glutamine, and 100 Units/mL of penicillin and 100 µg/mL of streptomycin. The culture was maintained at a temperature of 37°C in a 5% $CO_2$. The cells were cultured through regular sub-passaging at 3-day intervals.

### The Effect of LA on the Growth of *L. infantum* Promastigotes

The antileishmanial properties of LA essential oil against *L. infantum* were implemented using a resazurin assay, as introduced in Islek et al. (27). Briefly, promastigotes were cultured in 96-well plates at $3x10^5$ cells per well in a completed RPMI medium. The cells were subsequently treated with 0.063-1 µL/mL of LA essential oil and incubated for 24, 48, and 72 h. Following incubations, the resazurin solution (2.5 mM) was put into each well at a concentration of 10% (v/v), and luminescence intensity was assessed using a UV-Vis spectrophotometer with

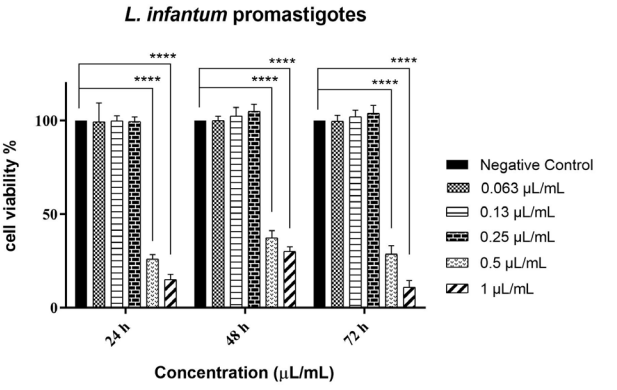


**Figure 1.** Effect of LA on the growth of *L. infantum* promastigotes. Viability of parasites following 24, 48, and 72 h of treatment with LA essential oil at various concentrations (0.063 to 1 µL/mL).

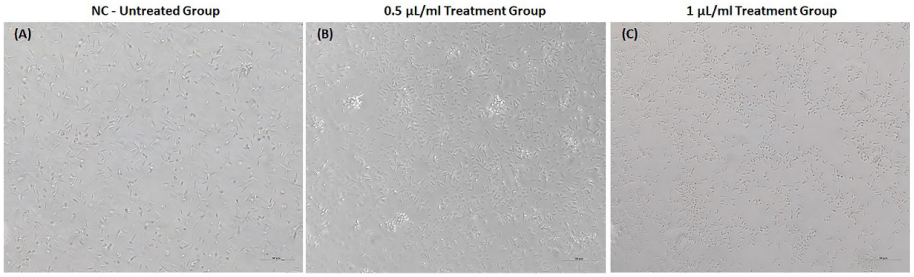


**Figure 2.** Phase-contrast microscopy images of **(A)** negative control (NC - untreated *L. infantum* promastigotes), **(B)** 0.5 µL/mL, and **(C)** 1 µL/mL LA essential oil treatment groups after 24 h of incubation. Bars correspond to 50 µm.

an excitation (560 nm) and an emission wavelength (590 nm). The viabilities (%) were determined relative to the negative control group, which was not subjected to any treatment. The data underwent analysis through the utilization of GraphPad Prism 8.01 software, following which the values for the 50% inhibitory concentration ($IC_{50}$) were determined.

## Cell Viability Assay on Macrophages

RAW264.7 macrophages were treated with LA essential oil at varying concentrations (0.063 to 1 µL/mL), and viabilities were conducted through the utilization of the resazurin assay. Accordingly, the RAW264.7 cells were harvested in 96-well plates at 10,000 cells per well. Following 24h, the cells were incubated with various concentrations (0.063-1 µL/mL) of LA essential oil for 24, 48, and 72 h. After treatments, the cells were subjected to a 2-h incubation period in a 2.5 mM resazurin solution with a concentration of 10% (v/v), and then the luminescence intensity was assessed by a ultraviolet-visible (UV-Vis) spectrophotometer. The cell viability (%) was determined according to the negative control group. The data underwent analysis using GraphPad Prism 8.01 software.

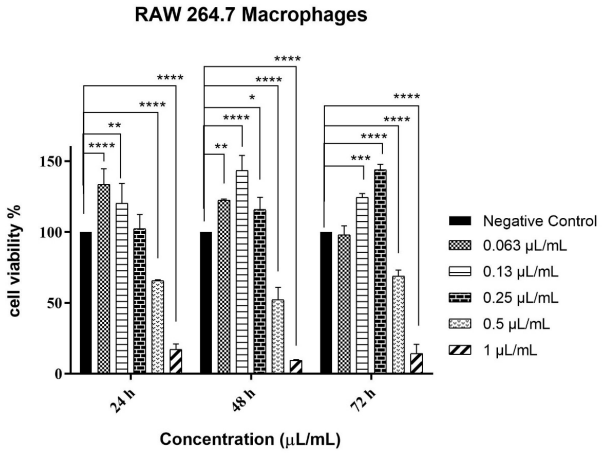


**Figure 3.** The viability analysis of RAW264.7 murine macrophages treated with LA essential oil at different concentrations (0.063-1 µL/mL) after 24, 48, and 72 h, compared to negative control.

## Statistical Analysis

The statistical analysis was conducted utilizing GraphPad Prism Software (version 8.01). The study used statistical analysis by conducting a comparison of data sets using a two-way ANOVA, followed by Tukey's multiple comparison test. The statistical significance of the findings were assessed at different levels of probability, *i.e.* (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

# RESULTS

## GC-MS Analysis

The GC-MS technique was utilized to determine the phytochemical composition of LA essential oil. As presented in Table 1, linalool was found to be the predominant constituent of the lavender oil at 37%, and it was consistent with the European Pharmacopoeia, in which linalool content should range from 20.0% to 45.0%. In addition, 1,8-Cineole, camphor, and limonene were detected in the lavender oil as 13.8%, 12.7%, and 2.4%, respectively (Table 1), which was found above the criteria of European Pharmacopoeia, whereas 7.7% linalyl acetate was detected as below the criteria. However, the percentage of the 4-terpinol content was found at 3.7%, which was consistent with the value within the range from 1.0 to 8.0% given in the European Pharmacopoeia. Likewise, the content of lavandulol and α-terpineol in lavender oil was detected at 0.5% and 1.5%, respectively, which confirms the quality criteria of obtained lavender oil considering the European Pharmacopoeia (the criteria for lavandulol: min. 0.1%; α-terpineol: max. 2.5%). The chemical quality of the oil utilized was found to be above the specified limitation regarding to the high linalool content (*i.e.,* 37%) (Table 1).

## Cell Culture Studies

### *In vitro* Antileishmanial Effect of the LA Essential Oil on *L. infantum* Parasites

The treatment groups of 0.5 and 1 µL/mL exhibited a dose and time-dependent inhibition of parasite proliferation. Treatment with 0.5 µL/mL LA essential oil significantly decreased promastigote viability to 26% ($p \leq 0.0001$) at 24 h, and became nearly 38% and

**Table 1.** Phytochemical composition and retention time (min) of the hydro-distilled LA essential oil using GC-MS analysis.

| *Retention index* | *Retention time (min)* | *Compounds* | *Area%* |
|---|---|---|---|
| *1027* | 7.98 | α-pinene | 1.5 |
| *1113* | 10.06 | β-pinene | 1.1 |
| *1212* | 12.63 | limonene | 2.4 |
| *1224* | 13.05 | 1,8-Cineole | 13.8 |
| *1498* | 21.98 | camphor | 12.7 |
| *1548* | 22.51 | linalool | 37.0 |
| *1569* | 22.61 | linalyl acetate | 7.7 |
| *1593* | 24.00 | 4-terpineol | 3.7 |
| *1679* | 25.65 | lavandulol | 0.5 |
| *1694* | 26.24 | α-terpineol | 1.5 |

29% at 48, and 72 h ($p \leq 0.0001$), respectively. Upon an increase in the concentration to 1 µL/mL, the viability of promastigotes exhibited a significant decrease to 15% (*i.e.*, 15.11%) after 24 h ($p \leq 0.0001$), and became nearly 30% (*i.e.*, 30.09%) and 11% (*i.e.*, 11.01%) at 48 h and 72 h, respectively, as depicted in Figure 1 ($p \leq 0.0001$)**.** However, there was no significant further alteration in the inhibition of the promastigote proliferation at concentrations lower than 0.5 µL/mL.

The $IC_{50}$ values were determined for LA essential oil following 24, 48, and 72 h of incubation, as 0.43 ± 0.089 µL/mL, 0.46 ± 0.053 µL/mL, and 0.47 ± 0.033 µL/mL, respectively. The findings demonstrated that 24, 48, and 72 h-incubation of LA essential oil exhibited efficacy against *L. infantum* promastigotes.

## Effects of LA Essential Oil on Morphology of *L. infantum* Parasites

In parallel to the parasite viability, phase-contrast microscopy images suggested that treatments with 0.5 and 1 µL/mL concentrations of LA essential oil indicated a dose-dependent reduction in the number of the parasite and transformation from elongated-shape to round-shaped morphologies at 24 h of incubation (Figure 2).

## Cytotoxicity of LA Essential Oil on Macrophages

After conducting an *in vitro* assessment of the antileishmanial efficacy of LA essential oil on *L. infantum* promastigotes, the cytotoxicity was assessed on healthy murine RAW264.7 macrophages (Figure 3). As shown in Figure 3, when murine macrophages were treated with LA essential oil at concentrations ≤ 0.25 µL/mL, cell viability of macrophages remained above 100%, and LA essential oil did not lead to toxicity on macrophages for 24, 48, and 72 h. Besides, the viability was increased to approximately 100%, 116% ($p \leq 0.05$), and 144% ($p \leq 0.0001$) following treatment with 0.25 µL/mL LA essential oil for 24, 48, and 72 h, respectively. Conversely, upon increasing concentration to 0.5 µL/mL, the viability was decreased to 66% at 24 h, which became 52% and 70% at 48, and 72 h of incubation, compared to negative control ($p \leq 0.0001$; Figure 3). A minor recovery in cell viability was detected among 48- and 72-h of treatment as ranging from 52% to 70% of cell viability. However, when the macrophages were treated with 1 µL/mL LA essential oil, the macrophage viability was decreased to below 17%, 10%, and 15% at 24, 48, and 72 h, respectively ($p \leq 0.0001$).

The results revealed that 0.5 µL/mL concentration of LA essential oil indicated a significant antileishmanial effect on *L. infantum* parasite proliferation with relatively less toxicity on macrophages.

## DISCUSSION

Leishmaniasis, a neglected disease, has affected a significant number of individuals globally and continues to pose significant challenges in terms of drug development. The complexity of the disease, including the difficulty of diagnosis, coupled with limited financial incentives for pharmaceutical companies, has hindered progress in finding effective treatments. Additionally, the available drugs suffer from drawbacks such as high toxicity, prolonged treatment durations, and emerging drug resistance. In contrast to synthetic pharmaceuticals, a greater abundance of natural drug resources exists, and the pursuit of novel drug discovery from natural products and traditional herbal medicine warrants serious consideration. In the present study, the plant-based, potential antileishmanial therapy was examined, such as LA, due to its high biocompatibility, natural properties, and low toxicity in RAW264.7 cells through mitochondrial respiration. In short, LA essential oil was examined by GC-MS

analysis to detect its composition, according to the European Pharmacopoeia, and following the GC-MS analysis, the cell culture studies, including the antileishmanial activity on *L. infantum*, and safety features of the compound on healthy macrophages were examined *in vitro* models.

Accordingly, the composition of LA essential oil was detected by the GC-MS technique, which was consistent with previous reports in the literature (19, 28). Linalool was found to be the main component of lavender oil (37%), which was linked to the characteristic compounds within the composition of LA essential oil, and consistent with the quality criteria of the European Pharmacopoeia (*i.e.*, 20.0 to 45.0%; Table 1). A previous study suggested that the presence of the key ingredients in lavender essential oil, such as linalool, and terpineol can enhance its penetration to the cell membrane (29, 30). Besides that, terpineol has the ability to bind to particular G protein-coupled receptors, leading to an impact on the cAMP and $Ca^{2+}$ concentrations, thus enhancing the activation of corresponding kinases, and ultimately facilitating the manifestation of its biological activities. Antileishmanial efficacy could be linked to the regulation of the kinase domain of calcium-dependent protein kinases (CDPKs) through calcium ions, and CDPK1 exhibits a significant association with the adhesion and invasion processes of protozoons (29).

In the present study, according to the growth inhibition on *L. infantum* promastigotes, the $IC_{50}$ values were calculated for LA essential oil as 0.47±0.033 µL/mL at 72 h, and 0.5 and 1 µL/mL treatment groups exhibited a dose-dependent inhibition of parasite proliferation (Figure 1). Additionally, RAW264.7 macrophage cytotoxicity studies revealed relatively no observed toxicity toward the host cells at concentrations equal to the $IC_{50}$ concentrations (Figure 3). Similarly, Yao et al. (2021) stated the antiprotozoal activity of LA essential oil against *Toxoplasma gondii* as dose-dependent within safe concentration ranges, and furthermore, the viability was not significantly reduced by LA essential oil at a 4.48 mg/ml in human foreskin fibroblasts (29). Furthermore, LA, and their nanoemulsions were found to be significantly effective on *L. major* parasites compared to *R. officinalis* essential oils, and conventional pentavalent antileishmanial chemotherapeutic, meglumine antimoniate (19). *Lavender* and *Rosemary* essential oils indicated the activity of *L. major* promastigote with $IC_{50}$= 0.11 µL/mL, and $IC_{50}$= 0.26 µL/mL, respectively, after 72 h of incubation (19). In parallel to viability studies, phase-contrast microscopy images demonstrated that 0.5 and 1 µL/mL treatments groups of LA essential oil indicated a dose-dependent reduction in the number of the parasite and transformation from elongated-shape to round-shaped morphologies at 24 h of incubation (Figure 2). The observed inhibitory effect could potentially be attributed to the impact on the morphology of *L. infantum* parasites, associated with the loss of membrane and flagellum integrity, and apoptosis. Nevertheless, the precise mechanism through which essential oils act on *L. infantum* remains uncertain and necessitates further investigation.

## CONCLUSION

In short, this study is the first statement on the antileishmanial activity of LA essential oil on *L. infantum* parasites. The present investigation provides preliminary studies of the traditional use of LA for antileishmanial therapy with *in vitro* cell culture analysis. The promising antileishmanial efficacy of lavender essential oil highlights the potential therapy in future *in vivo* studies.

**Acknowledgments:** We would like to thank Ezgi Taskan for her contribution to the cell viability studies.

**Ethics Committee Approval:** Ethics committee approval is not required because of no material or experimental animal that would require permission.

**Authors' Contributions:** Conception/Design of Study – F.S., Z.I.; Data Acquisition – F.S., Z.I.; Data Analysis/Interpretation – Z.I.; Drafting Manuscript– F.S., Z.I.; Critical Revision of Manuscript- F.S., Z.I.; Final Approval and Accountability– F.S., Z.I.

**Conflicts of Interest:** The authors did not disclose any potential conflicts of interest.

**Financial Disclosure:** This study was funded by the Department of Genetics and Bioengineering, Yeditepe University.

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Experimed 2023; 13(2): 121-126

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1308908

# Cryptotanshinone Protects Against Acute Pulmonary Edema

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**Cite this article as:** Yilmaz U, Demir M. Cryptotanshinone protects against acute pulmonary edema. Experimed 2023; 13(2): 121-126.

## ABSTRACT

**Objective:** Cryptotanshinone (CTS) is a compound with anti-inflammatory, anti-bacterial, anti-oxidative and anti-aggregant functions. We aimed to determine the effects of CTS on α-naphtilthiourea (ANTU)-induced acute pulmonary edema.

**Materials and Methods:** In this study, 4 groups (control, sham, ANTU and ANTU+CTS) were established from a total of 40 rats. The ANTU+CTS group received intraperitoneal CTS for seven days, and both ANTU and ANTU+CTS received an ANTU administration for the induction of peripheral effusion. Four hours after the ANTU administration, the rats were subjected to a forced swim test and were decapitated. Swimming times of rats, amount of pleural effusion (PE), lung weight (LW)/body weight (BW), and PE/BW ratios were determined.

**Results:** At the end of the experiment, PE was not detected in the lungs of control and sham group rats. It was determined that PE, LW/BW and PE/BW were significantly decreased, while swimming time was increased after acute pulmonary edema in the CTS group ($p<0.05$).

**Conclusion:** CTS showed a protective effect against acute pulmonary edema, which indicates that it may be used as a new therapeutic agent against pulmonary toxicity.

**Keywords:** Cryptotanshinone; α-naphthylthiourea, pulmonary edema, pleural effusion, swimming duration

## INTRODUCTION

Pleural diseases are extremely common today, affecting 3000 people out of every 1 million people annually. Pleural effusions (PE) constitute an important part of pleural diseases (1). It has been reported that approximately 90% of PE, which constitute an important part of pleural diseases, occur due to congestive heart failure, pneumonia, malignancy, and pulmonary embolism (2). Pulmonary edema occurs as a result of high pressure in the pulmonary microcirculation or an increase in the permeability of the alveolar-capillary barrier, or a combination of both (3). Pulmonary edema is an imbalance between the formation and reabsorption of lung tissue fluid, leading to massive tissue fluid reabsorption by pulmonary lymphatic and venous vessels insufficiency. Pulmonary edema can also be defined as abnormal fluid accumulation in the extravascular parts of the lung (4). Pulmonary edema is divided into two subgroups as cardiogenic and non-cardiogenic (5). Non-cardiogenic pulmonary edema occurs with increased alveolocapillary permeability as a direct or indirect result of a pathological process (multiple trauma, septic shock, influenza pneumonia, aspiration syndrome) (6). Cardiogenic pulmonary edema is characterized by increased secondary hydrostatic capillary pressure due to increased pulmonary venous pressure (7). Cardiogenic pulmonary edema leads to acute respiratory failure and has a high mortality rate (8).

Cryptotanshinone (CTS) is a lipophilic compound derived from the roots of the plant species *Salvia miltiorrhiza*, which is used in the treatment of cardiovascular diseases, hepatitis, diabetes, and chronic liver failure in China (9). This compound, which has the potential to prevent many

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**Submitted:** 02.06.2023 **Revision Requested:** 06.07.2023 **Last Revision Received:** 06.07.2023 **Accepted:** 11.07.2023 **Published Online:** 04.08.2023

diseases, also has anti-inflammatory, antibacterial, anticancer, antioxidative and antiaggregant effects (10-13). The important therapeutic effects of CTS in the treatment of angiogenesis-related diseases by inhibiting endothelial cell proliferation have been emphasized (14). CTS shows antipermeability and antiangiogenic effects, as well as anti-inflammatory properties, in interaction with other cytokines and chemokines (15). It has been suggested that since CTS alleviates pulmonary fibrosis (16), it is a promising drug candidate for the treatment of fibrotic pulmonary diseases (17). In addition, inhalation of CTS is a safe and effective treatment strategy for chronic pulmonary fibrosis. (18). Although CTS has anti-inflammatory effects against acute lung injury, it also has therapeutic effects (19). Moreover, it can efficiently protect pulmonary function and decrease early pulmonary inflammation infiltration in radiation-induced lung injury, and it was found to be more effective than prednisone in alleviating pulmonary fibrosis (20).

For the identification of new therapeutic agents in the treatment of pulmonary edema, the α-naphthylthiourea (ANTU)-induced pulmonary edema model is the most widely used and most similar to the clinicopathological features of pulmonary edema in humans (21). ANTU, a chemical compound derived from thiourea, was first used as a rodenticide, and it causes an increase in pulmonary vascular permeability in the rat, leading to the development of pulmonary edema (22).

To date, several antioxidants have been used as an alternative treatment for the cure of various diseases, including pulmonary edema. The potential of numerous flavonoids isolated from natural sources has been demonstrated against various lung diseases. However, the protective effect of CTS, which is a strong antioxidant substance against acute pulmonary edema, has not been studied so far.

We aimed to determine the effects of CTS on the amount of PE, lung weight (LW)/body weight (BW), and PE/BW ratios and forced swim test time in the lungs of rats with acute pulmonary edema with ANTU.

## MATERIALS AND METHODS

The study was performed with the approval of the Karabuk University Medical Faculty Experimental Animals Ethics Committee (Date: 04/10/2022, Decision number: 2022/09/15, Registration number: E-55212866-050.99-174960). A total of 40 male Wistar Albino rats with an average weight of 200-240 grams were included in the study. During the study, the rats were housed in a 12-hour light/12-hour dark cycle and a temperature of 21±2°C. The animals were fed *ad libitum* with standard rat pellet feed produced for experimental animals and were given tap water.

### Experimental Groups and Experimental Design

Before the experimental stages, all the rats were weighted, and experimental groups were formed from those whose body weights were closest to each other. Experimental groups: They were randomly divided into 4 groups as control, sham, ANTU and ANTU+CTS (n=10, for each group).

Control: Rats of this group were given CTS solvent (saline containing 1% Tween 80) in a volume of CTS for 7 days from day 1 of the study.

Sham: The rats in this group were given a single intraperitoneal ANTU solvent (olive oil) (Sigma-Aldrich, USA) dose volume of ANTU on the 7th day of the study.

ANTU: The rats of this group were injected with a single dose of ANTU intraperitoneal at a dose of 10 mg/kg dissolved in olive oil (4 mg/mL) on the 7th day of the study.

ANTU+CTS: The rats of this group were administered CTS (10 mg/kg) (Sigma-Aldrich, USA) dissolved in saline containing 1% Tween 80 for 7 days from the 1st day of the study (23-25). Again, the rats in this group were injected with ANTU on the 7th day of the study.

### Establishment of Experimental Lung Edema Model with ANTU

Severe pulmonary edema and lung damage were observed in rats injected with ANTU within 4 hours. As a result of this damage, PE formation, LW/BW and PE/BW ratios were found to be increased. Floatation exercises were performed on the rats 4 hours after the ANTU injection.

### Forced Swim Test

The forced swim test is commonly used to evaluate fatigue behavior in animal experiments. The pulmonary edema and effusion we created experimentally lead to fatigue and respiratory failure because of the decreased oxygenation capacity and lung compliance of the lungs of rats. The forced swim test was utilized to evaluate the effect of CTS on induced PE.

For the rats to get used to the pool, they were allowed to swim free for 15 minutes the day before the experiment and were dried and returned to their cages. After seven days of CTS and solvent injections, on the 7th day of the experiment (4 hours after the ANTU injection), the rats were subjected to a forced swim test in a plastic pool (90 cm × 45 cm × 45 cm) filled with room temperature water. In this test, the depth of the water should have been sufficient to prevent the animals from touching the floor of the pool with their tails. The swimming period was taken as the duration spent by the rats swimming until they exhausted their strength and started struggling. The end point of the strenuous swimming time was accepted as the point where the nose of the rats remained below the water surface for 10 seconds. When obvious signs of fatigue appeared, the animals were removed from the pool, and returned to their cages (26).

## Termination of Experiment

Following the swimming exercises, the rats were euthanized by taking blood from the abdominal aorta under anesthesia (intraperitoneal ketamine/xylazine (100 mg/kg / 5 mg/kg). The thorax was opened very carefully, and utmost care was taken not to make the surrounding tissues bleed and not to contaminate the effusion fluid with blood. The effusion fluid was collected from the pleural space with a syringe, and the lungs were carefully isolated and cleaned from the surrounding tissues. The removed lungs were weighed on a precision scale. The amount of PE, LW/BW, and PE/BW ratios were calculated.

## Statistical Analyses

Statistical analyses were performed with IBM SPSS 25.0 program. The normal distribution was determined with the Shapiro Wilk test. When the data of the groups did not show normal distribution, the difference between the groups was evaluated with the Kruskal Wallis H test. When statistical significance was detected between groups, multiple/pair comparisons were analyzed with the Bonferroni correction-Mann Whitney U test. $p<0.05$ was considered significant.

# RESULTS

## Effect of CTS on PE

It was detected that there was no PE in the lungs of the rats in the control and sham groups, when the study was terminated, and the thorax of rats were opened. Non-hemorrhagic PE with exudate characteristics was determined in the lungs of rats in the ANTU and ANTU+CTS groups. When the pleural effusions of groups were compared; it was determined that the amount of pleural fluid in the ANTU (4.1±0.5 mL) group and ANTU+CTS (1.8 ± 0.4 mL) groups were statistically significantly higher than in the control and sham groups ($p<0.05$). In addition, when the ANTU group and ANTU+CTS groups were compared, it was found that the pleural fluid of the ANTU+CTS group was statistically reduced compared to the ANTU group ($p<0.05$) (Figure 1). It can be said that CTS provides protection against the formation of PE.

## Effect of CTS on PE/BW × $10^4$

When the PE/BW ratios of the groups were compared; the PE/BW ratio of the ANTU (144.9 ± 3.8) and ANTU+CTS (83.6 ± 1.8) groups was statistically significantly higher than the control and sham groups ($p<0.05$). However, when ANTU and ANTU+CTS groups were compared in terms of PE/BW ratio, it was determined that CTS statistically significantly reduced the PE/BW weight ratio ($p<0.05$) (Figure 2).

## Effect of CTS on LW/BW × $10^4$

By the end of the experiment, when the LW/BW ratios of the groups were compared; the LW/BW ratio of the ANTU (93.6 ± 9.4) and ANTU+CTS (79.7 ± 3.8) groups was statistically significantly higher than the control (47.7 ± 2.6) and sham (46.3 ± 2.4) groups ($p<0.05$), while it was statistically lower in the ANTU+CTS group than ANTU ($p<0.05$) (Figure 3).

## Effect of CTS on Swimming Time

On the seventh day of the experiment, four hours after ANTU was given to ANTU and ANTU+CTS groups, all experimental groups were given a swimming test. Regarding the swimming times of the groups; it was statistically significantly lower in ANTU (18.8 ± 6.9) and ANTU+CTS (30.2 ± 5.8) than in the control (50.2 ± 5.1) and sham (48.4 ± 4.9) groups ($p<0.05$). Moreover, the swimming time of the ANTU+CTS group was statistically

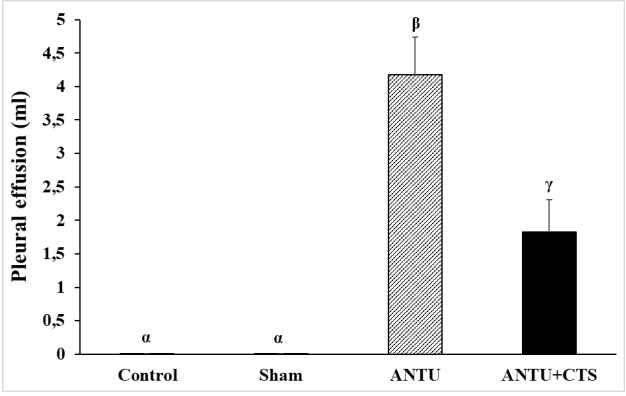


**Figure 1.** Effect of CTS on PE.
α:There was no significance between the control and sham groups.
β:The ANTU group is significantly higher than the control and sham groups.
γ: The ANTU+CTS group was significantly reduced compared to the ANTU group.

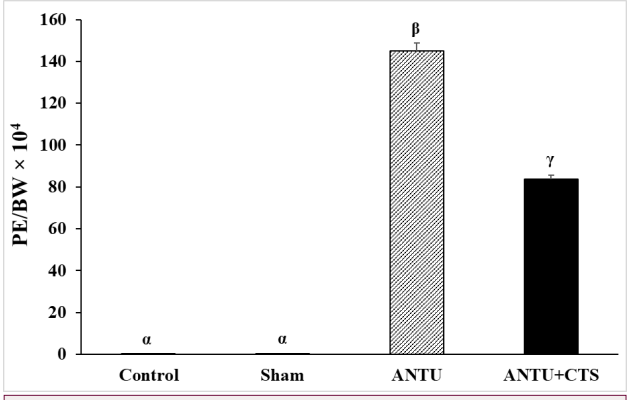


**Figure 2.** Effect of CTS on PE/BW × $10^4$.
α:There was no significance between the control and sham groups.
β:The ANTU group is significantly higher than the control and sham groups.
γ: The ANTU+CTS group was significantly reduced compared to the ANTU group.

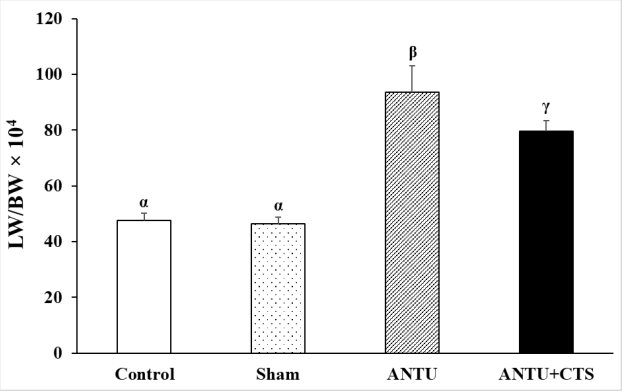


**Figure 3.** Effect of CTS on LW/BW.
α:There was no significance between the control and sham groups.
β:The ANTU group is significantly higher than the control and sham groups.
γ: The ANTU+CTS group was significantly reduced compared to the ANTU group.

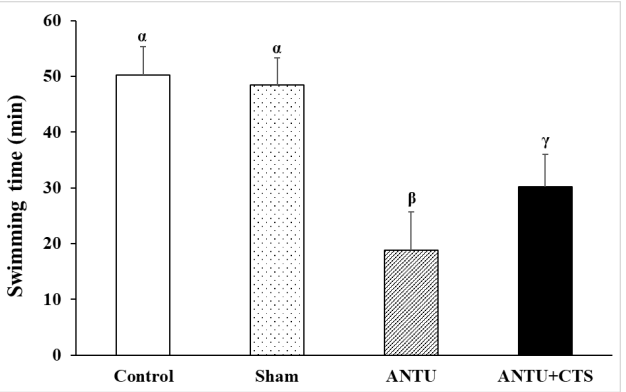


**Figure 4.** Effect of CTS on swimming time.
α:There was no significance between the control and sham groups.
β:The ANTU group is significantly lower than the control and sham groups.
γ: The ANTU+CTS group was significantly increased compared to the ANTU group.

higher ($p<0.05$) than ANTU (Figure 4). It can be said that CTS reduces the formation of PE and increases swimming time.

## DISCUSSION

ANTU gives rise to acute pulmonary vascular injury in rodents, with the most prominent manifestations including increased lung weight due to PE and pulmonary edema due to damage to endothelial cells and pneumocytes in the lung. Since the effects of ANTU are highly specific to the lungs, it is widely used in generating animal models to investigate the pathology of acute pulmonary edema (27). CTS, a key ingredient derived from *Salvia miltiorrhiza*, has many pharmacological properties such as anti-inflammatory, anti-oxidative, anti-angiogenic, and anti-proliferative properties. CTS is used in the treatment of many diseases including acute lung injury (18). Additionally, orally administered CTS is an alternative treatment agent for pulmonary fibrosis (17). However, its poor oral absorption and sensitivity to light greatly limit its clinical application (28).

Regarding its anti-cancer properties, it has been stated that CTS induces apoptosis in lung cancer cells and inhibits tumor formation in the lung both *in vitro* and *in vivo* (29). Furthermore, CTS prevents invasion/metastasis in non-small cell lung cancer (NSCLC) via inhibiting the expression of carcinogenic microRNAs (30, 31). It has also been stated that CTS potentiates the effects of chemotherapeutic drugs on NSCLC cells through transketolase inhibition both *in vitro* and *in vivo* (32). When this information is compiled, it can be concluded that CTS has suppressive effects on lung cancer, can be used as a treatment agent in *in vivo* and *in vitro* studies.

It has been stated that CTS reduces allergic airway inflammation (33), and might be a curative agent for the treatment of asthma (34). Moreover, CTS has been shown to have an anti-inflammatory effect by down-regulating pro-inflammatory inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the mouse macrophage cell line (35). In addition, CTS also performed anti-inflammatory effects specifically by inhibiting COX-2 activity in lipopolysaccharide (LPS)-stimulated pro-monocyte cell line and paw edema model (13). Tang et al. have shown that CTS reduces LPS-induced acute lung injury through its anti-inflammatory effects by inhibiting toll like receptor 4 (TLR4)-mediated nuclear factor kappa B (NF-kB) signaling pathways (36). Moreover, CTS maintain to pulmonary fibrosis by suppressing the cascade of Smad and STAT3 (37). CTS treatment has been shown to reduce radiation-induced lung injury and pulmonary fibrosis in rats by suppressing inflammatory and fibrotic factors (38). Similarly, its inhalation has also been shown to alleviate pulmonary fibrosis; therefore, CTS may be an alternative treatment method in the treatment of pulmonary fibrosis (39).

To date, the effects of CTS have not been examined on pulmonary edema. However, considering the studies on CTS, its protective role in pulmonary diseases and edema is due to its anti-inflammatory effects through inhibition of the inflammatory pathway. In this study, it was determined that the use of CTS improves swimming scores by reducing pleural fluid in the ANTU-induced pulmonary edema model. CTS may have shown a protective effect against pulmonary edema by inhibiting the anti-inflammatory pathway.

## CONCLUSION

Our findings have demonstrated that CTS shows a protective effect against ANTU-induced acute pulmonary edema. CTS decreased PE, LW/BW, and PE/BW following pulmonary edema, while increasing swimming time. CTS seems like it may be considered as an alternative treatment approach in the treatment of pulmonary edema following more detailed

studies. The lack of molecular analyses is one of the limiting factors of our study, however, it provides important preliminary information for studies that will attempt to understand the effects of CTS on pulmonary edema with molecular analysis.

**Ethics Committee Approval:** This study was conducted with the approval of Karabuk University Faculty of Medicine Experimental Animals Ethics Committee (Date: 04/10/2022, Decision Number: 2022/09/15, Registration number: E-55212866-050.99-174960).

**Authors' Contributions:** Conception/Design of Study- U.Y., M.D.; Data Acquisition – U.Y., M.D.; Data Analysis/Interpretation – U.Y., M.D.; Drafting Manuscript– U.Y.; Critical Revision of Manuscript- U.Y.; Final Approval and Accountability– U.Y., M.D.

**Conflict of Interest:** Authors declared no conflict of interest.

**Financial Disclosure:** Authors declared no financial support.

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Experimed 2023; 13(2): 127-132

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1319722

# Comparative Toxicity Responses of Thirdhand Smoke Derived from Conventional Cigarette and Heated Tobacco Products in Human Bronchial Epithelial Cells

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**Cite this article as:** Reis R, Kolci K. Comparative toxicity responses of thirdhand smoke derived from conventional cigarette and heated tobacco products in human bronchial epithelial cells. Experimed 2023; 13(2): 127-132.

## ABSTRACT

**Objective:** Thirdhand smoke (THS) is described as the accumulated chemicals left on indoor surfaces after tobacco smoking. Individuals can be exposed to THS by dermal or oral contact with THS-embedded surfaces or by breathing in the off-gasses. In the present study, the cytotoxic, oxidative, and inflammatory responses of THS extracts yielded from conventional (THS-C) and heated-tobacco products (THS-H) were examined in BEAS-2B human bronchial epithelial cell line.

**Materials and Methods:** The terrycloth samples were exposed to smoke in a closed polystyrene box and extracted in a complete cell culture medium for 24 hours at 37°C. Following this, the cytotoxicity of THS was assessed by MTT assay. Malondialdehyde (MDA) and intracellular glutathione (GSH) levels were determined in BEAS-2B cell lysate to assess oxidative response. The aryl hydrocarbon receptor (AhR) and interleukine-6 (IL-6) levels were determined via an ELISA kit.

**Results:** Both types of THS led to dose-dependent cytotoxicity in cells, which was remarkable with THS-C (50%, v/v). Moreover, GSH depletion and MDA increase were remarkable with both THS, particularly with THS-C. AhR activation was also slightly elevated with THS-C, whereas the increase in IL-6 was notable compared to THS-H.

**Conclusion:** THS exposure might lead to potential health risks particularly for respiratory health and the results support the need for comprehensive regulations and public health initiatives to minimize the harmful effects of THS.

**Keywords:** Thirdhand smoke, heated-tobacco product, oxidative stress, aryl hydrocarbon receptor, inflammation, interleukine-6.

## INTRODUCTION

Smoking is one of the leading causes of preventable death worldwide. Across Organisation for Economic Co-operation and Development (OECD) countries, among smokers aged 15 and over, Turkiye is ranked second with a daily smoking rate of 28% after Indonesia, according to the latest data (1). Due to this relatively high rate of daily smoking, other risk factors may arise not only for active cigarette smokers but also for non-smokers and the environment exposed to the side stream smoke during this process (2). From this point of view, a new toxicological concept, thirdhand smoke (THS), can be described as residual smoke that is remained after the cigarette is extinguished and might be generated from aged secondhand smoke that adheres to dust and surfaces. In addition to its cumulative hazard potential, its re-emission into the air makes the THS a public health concern, particularly for individuals who are suffering from respiratory diseases (3). Even though smoking has been prohibited in most indoor workplaces and public places in Turkiye since 2005, there are a few exceptions where ventilation is allowed for smoking such as care facilities for the elderly people, restricted areas in airports, personal accommodation places, and hotel rooms (4). Therefore,

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**Submitted:** 25.06.2023 **Revision Requested:** 18.07.2023 **Last Revision Received:** 18.07.2023 **Accepted:** 27.07.2023 **Published Online:** 31.07.2023

indoor smoking might represent a current public health and environmental concern for people who do not actively smoke, despite strengthened bans.

According to the recent literature, the chemical dynamic of THS is quite different from firsthand smoke (FHS) and secondhand cigarette smoke (SHS) due to the delayed and aged exposure to smoke pollutants. In addition, the re-emitted gas phase represents a continuous environmental pollutant reservoir in terms of nicotine, nitrous acid, carcinogenic tobacco-specific nitrosamines (TSNAs), and volatile organic components (VOCs) (5). Unfortunately, physical, and chemical cleaning techniques were shown to be ineffective in removing THS residue adsorbed on surfaces and materials. Also, cleaning methods such as wiping was previously reported to cause particles to become liberated from the deposited surfaces and re-suspended in air, thus increasing the probability of exposure via the inhalation route (6). Exposure via inhalation is a common exposure route for FHS, SHS, and THS; however, due to the surface embedding potential of THS, the oral route is another exposure route for THS, which makes THS a health hazard for toddlers and children who are frequently in contact with surfaces via hand to mouth transfer with a long-lasting effect (7). In addition to the risk to children, people who are suffering from chronic respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and wheezing (8). According to research by Vanker and colleagues (2017), environmental cigarette smoke was suggested as an important public health concern for people, especially children, by increasing the rate of asthma, wheezing, and pulmonary infections. In addition, these health hazards were reported to be at higher rates in low and moderate-income countries in several reports (8,9).

The limited studies on THS exposure showed that it has dose-dependent cytotoxic (10) and genotoxic (11,12) potential on several cell lines and has a delaying effect on wound healing capacity in mice (13). Based on these few findings on the risk of repeated exposure to THS, a toxicological evaluation of the complex mixture of THS and its possible health effects are further needed. In recent years, in addition to the use of conventional cigarettes, "*less harmful*" options to smoke were started to be marketed such as heat-not-burn products or heated tobacco products (HTP). These products aim to serve a smoke containing nicotine to the consumer with a less burning product and carcinogenic intermediates since HTPs are heating the tobacco up to 300°C instead of burning as in traditional cigarettes (14,15). In our previous study, we reported the comparative cytotoxicity of the FHS extracted from HTP and conventional cigarette (3R4F) in human liver epithelial cells, and those novel products dose-dependently might be cytotoxic to the cells via inflammatory and oxidative pathways (16). However, there is no current finding on the residual potential of HTPs and their toxicity potential on the primary target organ, the lungs. Since there is no classified safety level of environmental cigarette smoke exposure, no definitive THS threshold for adverse effects has been recognized. The present study aimed to evaluate the potential cytotoxicity profile of two different types of THSs isolated from conventional cigarette and heated tobacco products preliminarily in human bronchial epithelial cells via oxidative and inflammatory pathways *in vitro*.

## MATERIAL AND METHODS

### Preparation of THS Extract

THS was extracted from terrycloth fabric exposed to one 3R4F cigarette (THS-C) and heat stick heated via HTP (THS-H) device manually in a polystyrene chamber with a puff volume of 55 mL and 30 seconds of puff cycle as described in the previous smoking simulation (16). After the smoke exposure in the chamber, the terrycloth was extracted in the high glucose Dulbecco`s Modified Eagle Media (DMEM, Gibco, USA) at 37°C for 24 hours. After the extraction process, the extract (100%, v/v) was filtered and stored at -80°C for further studies. The standardization of prepared extract was recorded through before and after weights of filter papers accumulates the total particulate matter (TPM) for each.

### Cytotoxicity Assay

The cytotoxicity potential of the two types of THSs was assessed via 3-(4,5-dimethylthiazol-2-yl)−2,5 diphenyltetrazolium bromide (MTT) assay through mitochondrial reductase activity in human bronchial epithelial BEAS-2B cells (ATCC, CRL-9609). For this purpose, cells were cultured with Dulbecco's modified eagle medium (DMEM) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 5% (v/v) fetal bovine serum (FBS, Gibco, USA). The cells were seeded in 96-well plates before the day of exposure as $2x10^4$ cells/well and incubated for 24 hours to achieve subconfluency. The following day, THS-C and THS-H were diluted with a complete cell culture medium and applied to the cells between doses at 10-100% (v/v) for 24 hours. After the incubation, the well contents were discarded and 0.5 mg/mL MTT solution was applied to the wells as previously described (17). In addition to the cytotoxicity, cell morphology was also visualized via EVOS M5000 (ThermoScientific, Singapour) at a magnification rate of 10x for 72 hours.

### Oxidative Stress

For the assessment of oxidative damage induced by THS-C and THS-H, intracellular glutathione (GSH) and lipid peroxidation intermediate product malondialdehyde (MDA) levels were assessed according to our previous methods (16,17). Briefly, the cells seeded in T-25 flasks were exposed to the 10-25-50% (v/v/v) THS-C and THS-H extracts for 24 hours. The next day, cell pellets were collected and lysed with MagnaLyser (Roche, Switzerland). The collected cell lysates were used for the assessment of total protein content, GSH, and MDA level spectrophotometrically in triplicates (ThermoScientific, VarioskanLUX, Singapour).

### Inflammatory Damage

Inflammation is another toxicity response against air pollutants; thus, it was evaluated in BEAS-2B cells via pro-inflammatory cytokine interleukin-6 (IL-6) and aryl hydrocarbon receptor

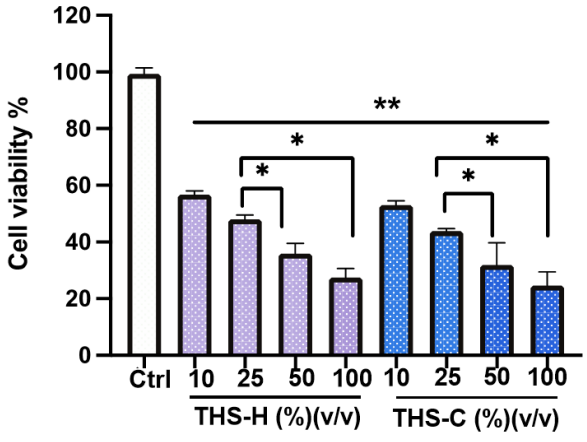


**Figure 1.** Cytotoxicity profiles of THS-C and THS-H in BEAS-2B cells.
Ctrl: Control; THS-H: Thirdhand smoke of HTP; THS-C: Thirdhand smoke of 3R4F cigarette. Results were expressed as mean± SD. The significant differences between groups and Ctrl were defined as *p<0.05; **p<0.001.

(AhR) levels via enzyme-linked Immuno sorbent assay (ELISA) kit. For this purpose, cell supernatants were assessed with a human IL-6 ELISA kit (Elabscience, E-EL-H6156, USA) and a human AhR ELISA kit (AFG Bioscience, EK700338, USA) at 450 nm spectrophotometrically in duplicates.

## Statistical Analyses

Statistical analyses of data were performed with GraphPad Prism 9.0 (La Jolla, California, USA). The results of cell culture studies are presented as the mean ± SD as triplicates. One-way analysis of variance (ANOVA) tests were used for analysis and differences were considered to be significant at p<0.05.

# RESULTS

## TPM of THS Extracts

The prepared extracts were standardized through collected TPM amounts on the filter papers. Based on our extractions, the TPM of THS-C was recorded as 3.7 ± 0.12 mg whereas for THS-H was 3.1 ± 0.2 mg.

## Cytotoxicity

Cytotoxicity of THS-C and THS-H were shown in Figure 1. As seen in Figure 1, both extracts showed dose-dependent cytotoxicity in BEAS-2B cells, which was remarkable with the higher doses of THS-C (50-100%, v/v). Furthermore, all the tested concentrations of THS-C and THS-H were significantly cytotoxic compared to the control group (p<0.001). According to the MTT assay, the inhibiting concentration 50% ($IC_{50}$) values of extracts were found as 14.8 ± 1.9% for THS-C and 21.7 ± 2.6% for THS-H. The cell morphology of THS-exposed BEAS-2B cells was recorded for 72 hours and shown in Figure 2.

## Oxidative Stress

Smoking is a well-known source of free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) formation, which play a crucial role in oxidative damage in target organs as a notable contributing factor. Hence, we assessed THS induced oxidative damage, which was yielded by a cigarette, and an HTP through intracellular GSH and MDA levels. Based on our findings, 25-50% (v/v) doses of THS-C and THS-H significantly led to a significant depletion in GSH deposits in BEAS-2B cells compared to the control (Figure 3). Moreover, the difference between the highest tested doses was remarkable with THS-C in the GSH assay (p<0.05). On the other hand, lipid peroxidation was significantly higher with THS-C (50%, v/v) compared to the same dose of THS-H (Figure 4) (p<0.001).

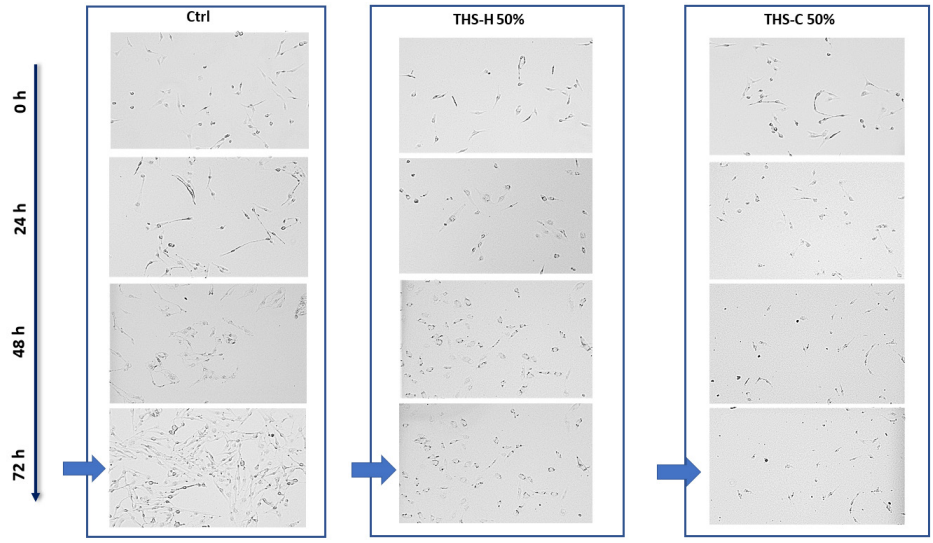


**Figure 2.** Morphological images of BEAS-2B cells exposed to the THS-C and THS-H for up to 72 hours.

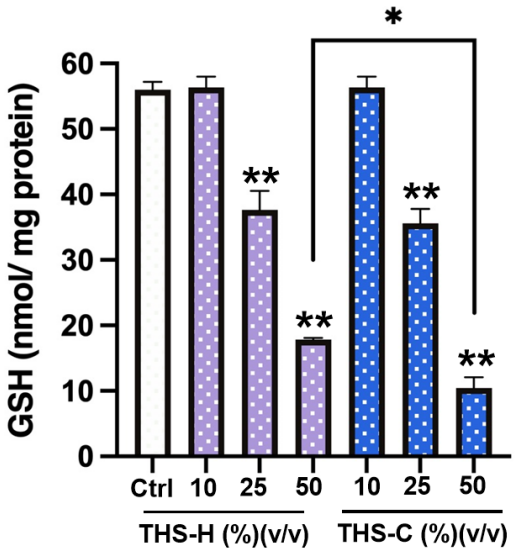


**Figure 3.** Intracellular GSH level in BEAS-2B cells.
Ctrl: Control; THS-H: Thirdhand smoke of HTP; THS-C: Thirdhand smoke of 3R4F cigarette. Results were expressed as mean± SD. The significant differences between groups and Ctrl were defined with * p< 0.05, **p<0.01

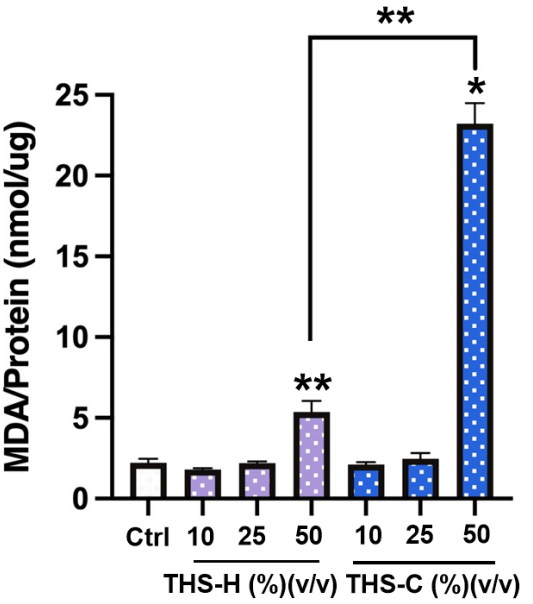


**Figure 4.** MDA level in BEAS-2B cells.
Ctrl: Control; THS-H: Thirdhand smoke of HTP; THS-C: Thirdhand smoke of 3R4F cigarette. Results were expressed as mean± SD. The significant differences between groups and Ctrl were defined with *p<0.05; **p<0.001

## Inflammatory Response

### IL-6 Levels

Inflammatory response through pro-inflammatory IL-6 level was found to be elevated with THS-C and THS-H exposure dose-dependently as shown in Figure 5. Based on the ELISA results, 50% and 100% (v/v) doses of the extracts exhibited a significant increase in the inflammatory response, which was the highest with THS-C. In addition, the difference in the IL-6 release was statistically significant between the highest tested doses of THSs (p<0.05).

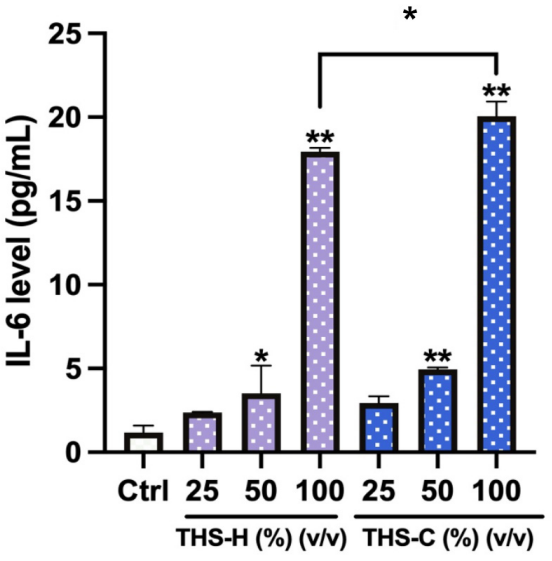


**Figure 5.** IL-6 level in BEAS-2B cells.
Ctrl: Control; THS-H: Thirdhand smoke of HTP; THS-C: Thirdhand smoke of 3R4F cigarette. Results were expressed as mean± SD. The significant differences between groups and Ctrl were defined with *p <0.05, **p<0.001.

### AhR Levels

Another important inflammatory marker, AhR, which has specific ligands such as tobacco smoke cigarette smoke carcinogen benzo(α)pyrene, was evaluated in BEAS-2B cells via ELISA kit. According to the present findings, cytoplasmic AhR level was higher with THS-H (100%, v/v) exposure compared to the same dose of THS-C (Figure 6). However, the difference between the two groups was insignificant.

## DISCUSSION

In the present study, we focused on the potential cytotoxicity, oxidative stress, and inflammatory response caused by THS derived from conventional cigarettes and heated tobacco products. The findings indicate that both THS-C and THS-H showed dose-dependent cytotoxicity in BEAS-2B cells, where the higher doses of THS-C exhibited more pronounced cytotoxic effects compared to THS-H based on their $IC_{50}$. Moreover, the cell morphology analysis further supported the cytotoxic effects of THS on the cells. Similar to our findings, Bahl and colleagues reported a dose-dependent cigarette-derived-THS cytotoxicity in human dermal fibroblasts (hDF)

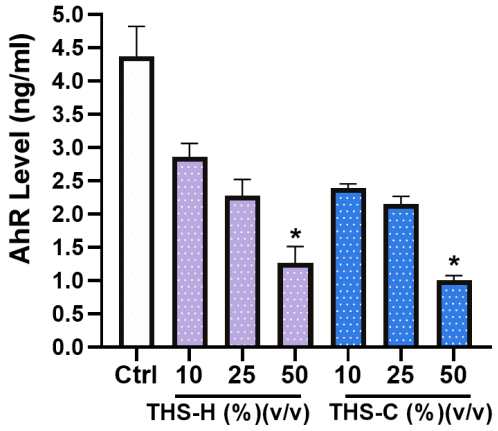


**Figure 6.** Cytoplasmic AhR level in BEAS-2B cells.
Ctrl: Control; THS-H: Thirdhand smoke of HTP; THS-C: Thirdhand smoke of 3R4F cigarette. Results were expressed as mean± SD. The significant differences between groups and Ctrl were defined with * p<0.001.

and human palatal mesenchyme cells (hPM) (10). In another study, indoor surface-derived THS was also found dose-dependently cytotoxic to mouse neural stem cells (mNSC), which represented a model for the neonatal brain (18). However, there are no records in the literature on the HTP-derived THS and potential cytotoxicity profile. Therefore, this is the first comparative report that identifies the cytotoxicity profile of HTP-derived THS *in vitro*. Oxidative stress is another important pathway involved in chronic pulmonary diseases and tobacco-induced lung deficits (19–21), and its assessment revealed that both THS-C and THS-H led to a depletion in intracellular GSH deposits in BEAS-2B cells. THS-C induced higher levels of lipid peroxidation compared to THS-H exposure, indicating its stronger potential for oxidative damage based on our findings. The other important pathway involved in respiratory diseases is inflammation, which was augmented with smoking as well, and was evaluated with pro-inflammatory IL-6 and tobacco-specific carcinogen ligand-induced-AhR level (22-25). According to the results, THS-C and THS-H exposure exhibited a dose-dependent increase in IL-6 levels, with THS-C showing a higher inflammatory response. However, there was no significant difference in cytoplasmic AhR levels between THS-C and THS-H. Previously, we reported a slight increase in HepG2 cells exposed to the FHS and SHS extracts of both conventional cigarettes and HTP, which was higher with cigarettes (16). Similarly, THS derived from a cigarette led to a higher AhR activation with a lower cytoplasmic AhR level in the current study. Based on these findings, it might be suggested that both types of THS demonstrated dose-dependent cytotoxicity, with THS-C exhibiting stronger effects. The oxidative stress assessment indicated a depletion of intracellular GSH deposits and higher lipid peroxidation with THS-C. Furthermore, THS-C induces a stronger inflammatory response through IL-6 release in BEAS-2B cells.

## CONCLUSION

These findings emphasize the potential health risks associated with THS exposure, particularly in terms of respiratory health. The study contributes to the understanding of the toxicological profile of THS and highlights the importance of further research to assess the long-term effects of THS exposure on human health. The results support the need for comprehensive regulations and public health initiatives to minimize THS exposure and protect individuals, especially those with respiratory conditions, from the harmful effects of THS.

**Acknowledgments:** This study was presented as an oral presentation at the 11th International Turkish Society of Toxicology Congress, Antalya, Turkiye (2-5 November 2022).

**Ethics Committee Approval:** Ethics committee approval is not required for cell culture studies in the article.

**Authors' Contributions:** Conception/Design of Study- K.K., R.R.; Data Acquisition – K.K., R.R.; Data Analysis/Interpretation – K.K., R.R.; Drafting Manuscript– R.R.; Critical Revision of Manuscript- R.R.; Final Approval and Accountability– K.K., R.R.

**Conflict of Interest:** Authors declared no conflict of interest.

**Financial Disclosure:** Authors declared no financial support.

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Experimed 2023; 13(2): 133-141

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1322111

# Pressure Ulcers in Palliative Care Unit Patients

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**Cite this article as:** Degerli MS. Pressure ulcers in palliative care unit patients. Experimed 2023; 13(2): 133-141.

## ABSTRACT

**Objective:** Pressure ulcers are associated with prolonged lengths of stay, increased costs, and lower discharge rates in palliative care units. This study aimed to reveal pressure ulcers' risk factors, incidence, and prevalence in a palliative care unit.

**Materials and Methods:** The data of 252 inpatients in a secondary-level palliative care unit were examined retrospectively. Patients were divided into groups according to pressure ulcer status, and data was comparatively analyzed. Pressure ulcer prevalence and incidence in the palliative care unit were calculated.

**Results:** There were 137 (54.4%) males and 115 (45.6%) females. The mean age was 67.4 ± 16.7. Most of them were oncology patients (30.6%) and cerebrovascular patients (30.6%). Pressure ulcers' prevalence and incidence were 40.1% and 4.7%, respectively. The Braden score was a median of 13 (5-23). In patients with pressure ulcers, the Braden score and discharge home rate were significantly lower; admission from another intensive care unit, the length of stay, and the treatment costs were significantly higher.

**Conclusion:** Pressure ulcers negatively affect the patient's length of stay in the palliative care unit, the discharge rate to home, and the financial burden on the healthcare system. Pressure ulcer risk analysis and a standardized care plan to prevent and treat pressure ulcers should be performed for cost-effective palliative care.

**Keywords:** Palliative care, pressure ulcer, healthcare cost, prevalence, incidence

## INTRODUCTION

With a prolonged life expectancy, increased prevalence of chronic diseases and cancer consequently increased the need for rehabilitation and palliative care. The World Health Organization defines “palliative care” as the relief of pain and other health problems in patients and families facing problems arising from a life-threatening illness (1). Palliative care does not aim to find curative treatment for diseases but to provide the management and control of symptoms and improve the comfort and life quality for the patient and his family in the last period of life (2).

A pressure ulcer is one of the most challenging problems to manage and control in palliative care units. They are lesions that occur on the skin or subcutaneous tissues due to pressure or friction and are often seen on bony prominences but can develop anywhere on the body (3, 4). Factors such as advanced age, immobilization, decreased sensorimotor sensation, a deterioration of nutritional status, and a decreased walking ability are essential in developing pressure ulcers. In addition, the development of pressure ulcers may indicate circulatory failure that occurs in the last period of life and gradually deepens (5, 6). In this respect, all palliative care unit patients are candidates for pressure ulcer development.

Pressure ulcers are associated with prolonged hospital stays, increased costs, and lower discharge rates. So pressure ulcer frequency and risk factors, a well-known issue in intensive care units, should also be examined in palliative care units.

This study aimed to reveal the factors affecting pressure ulcer development, a critical problem in the palliative care unit, and pressure ulcer incidence and prevalence in the palliative care unit.

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**Submitted:** 03.07.2023 **Revision Requested:** 21.07.2023 **Last Revision Received:** 21.07.2023 **Accepted:** 05.08.2023 **Published Online:** 07.08.2023

## MATERIALS AND METHODS

The study's ethical approval was obtained by the local ethics committee of Haseki Training and Research Hospital (Approval number: 71-2022).

The data of 252 patients who received inpatient treatment in a secondary-level state palliative care unit between January 2016 and January 2022 were analyzed retrospectively.

The Braden scale was used in the risk assessment of patients for pressure ulcers (7). In the analysis, patients' admission Braden scores were considered. European Pressure Ulcer Advisory Panel (EPUAP), National Pressure Injury Advisory Panel (NPIAP), and Pan Pacific Pressure Injury Alliance (PPPIA) guidelines were used for staging pressure ulcers (3). Pressure ulcers of the patients were evaluated daily and staged. Patients with no pressure ulcers hole the palliative care period was included in group-1. Complete recovery of the existing pressure ulcer, or getting

**Table 1.** Demographic data of the patients

| | | Min-max | Median | Mean±SD |
|---|---|---|---|---|
| Age (Year) | | 18.0 - 97.0 | 69.5 | 67.4 ± 16.7 |
| | | | **N** | **%** |
| Sex | Male | | 137 | 54.4 |
| | Female | | 115 | 45.6 |
| Comorbidity | Diabetes mellitus | | 34 | 13.5 |
| | Hypertension | | 44 | 17.5 |
| | Heart disease | | 29 | 11.5 |
| | Pulmonary disease | | 14 | 5.6 |
| | Renal failure | | 1 | 0.4 |
| Admission to palliative care from | House | | 123 | 48.8 |
| | Other hospital | | 63 | 25.0 |
| | Intensive care unit | | 66 | 26.2 |
| Diagnosis | Oncology | | 77 | 30.6 |
| | Cerebrovascular disease | | 77 | 30.6 |
| | Alzheimer/dementia | | 22 | 8.7 |
| | Hypoxic brain | | 19 | 7.5 |
| | Traumatic brain injury | | 17 | 6.7 |
| | Senility | | 16 | 6.3 |
| | Parkinson | | 8 | 3.2 |
| | Other | | 16 | 6.3 |
| Cancer type | Colon | | 11 | 14.3 |
| | Gastric | | 11 | 14.3 |
| | Breast | | 8 | 10.4 |
| | Lung | | 8 | 10.4 |
| | Brain | | 8 | 10.4 |
| | Pancreas | | 6 | 7.8 |
| | Bladder | | 4 | 5.2 |
| | Endometrium | | 3 | 3.9 |
| | Rectum | | 3 | 3.9 |
| | Renal | | 2 | 2.6 |
| | Ovary | | 2 | 2.6 |
| | Cholangiocarcinoma | | 2 | 2.6 |
| | Cervix | | 2 | 2.6 |
| | Hepatocellular | | 1 | 1.3 |
| | Larynx | | 1 | 1.3 |
| | Nasopharynx | | 1 | 1.3 |
| | Esophagus | | 1 | 1.3 |
| | Prostate | | 1 | 1.3 |
| | Gallbladder | | 1 | 1.3 |
| | Salivary gland | | 1 | 1.3 |

downstage, or decreasing the number of pressure ulcers were accepted as pressure ulcer healing, and these patients formed group-2. Patients whose pressure ulcers did not heal were divided into two groups; the patients with stable pressure ulcers (group-3) and those with worsening pressure ulcers (group-4). Patients with worsening pressure ulcers (group-4) consisted of

**Table 2.** Pressure ulcer related patients data

| | | Min-max | Median | Mean ± SD |
|---|---|---|---|---|
| Braden score | | 5.0-23.0 | 13.0 | 13.0 ± 2.7 |
| Length of stay | | 1.0-323.0 | 22.0 | 39.7 ± 51.6 |
| Cost of hospitalization (dollar) | | 82.7-100146 | 3742.1 | 8656.6 ± 13889.4 |
| | | | N | % |
| Pressure ulcer | Present | | 101 | 40.1 |
| | None | | 151 | 59.9 |
| Patients with pressure ulcer | Healed | | 20 | 19.8 |
| | Did not healed | | 81 | 80.2 |
| Patients whose pressure ulcers did not healed | Up staging pressure ulcers | | 8 | 9.9 |
| | Stable pressure ulcers | | 61 | 75.3 |
| | Added new pressure ulcers | | 12 | 14.8 |
| Multiple pressure ulcers at admission | Present | | 49 | 48.5 |
| | None | | 52 | 51.5 |
| Pressure ulcer localization at admission | Sacrum | | 37 | 36.6 |
| | Trochanter | | 2 | 2.0 |
| | Ischium | | 4 | 4.0 |
| | Heel | | 1 | 1.0 |
| | Scapula | | 7 | 6.9 |
| | Hand | | 1 | 1.0 |
| | Multiple localization | | 49 | 48.5 |
| Pressure ulcer localization at discharge | Sacrum | | 34 | 33.7 |
| | Trochanter | | 4 | 4.0 |
| | Ischium | | 4 | 4.0 |
| | Heel | | 3 | 3.0 |
| | Scapula | | 6 | 5.9 |
| | Hand | | 1 | 1.0 |
| | Multiple localization | | 49 | 48.5 |
| Vacuum assisted closure | Present | | 18 | 7.1 |
| | None | | 234 | 92.9 |
| Discharge status | To home | | 88 | 34.9 |
| | To intensive care unit | | 69 | 27.4 |
| | Transferred | | 75 | 29.8 |
| | Died | | 19 | 7.5 |
| | Nursing home | | 1 | 0.4 |

patients whose pressure ulcers were getting up stage, or a new one was added to the existing pressure ulcer.

Groups;

group-1: patients with no pressure ulcers,

group-2: patients with healing pressure ulcers,

group-3: patients with stable pressure ulcers,

group-4: patients with worsening pressure ulcers,

Daily skincare and standard care plans were applied to all patients with pressure ulcers.

**Table 3.** Comparative analysis of patients with pressure ulcers and those without

| | | Pressure ulcer (+) | | Pressure ulcer (-) | | p |
|---|---|---|---|---|---|---|
| | | Mean ± SD | Median | Mean ± SD | Median | |
| **Age (Year)** | | 69.6 ± 17.1 | 73.0 | 65.8 ± 16.3 | 67.0 | 0.050[a] |
| **Braden score** | | 11.2 ± 1.9 | 11.0 | 14.1 ± 2.5 | 14.0 | 0.000[a] |
| **Length of stay** | | 51.5 ± 57.8 | 29.0 | 31.7 ± 45.5 | 16.0 | 0.000[a] |
| **Cost of hospitalization (dollar)** | | 10520 ± 13301 | 5155 | 7410 ± 14177 | 3014 | 0.000[a] |
| | | **N** | **%** | **N** | **%** | **p** |
| **Sex** | Male | 53 | 52.5 | 84 | 55.6 | 0.622[b] |
| | Female | 48 | 47.5 | 67 | 44.4 | |
| **Comorbidity** | | | | | | |
| Diabetes mellitus | | 17 | 16.8 | 17 | 11.3 | 0.204[b] |
| Hypertension | | 22 | 21.8 | 22 | 14.6 | 0.139[b] |
| Heart disease | | 10 | 9.9 | 19 | 12.6 | 0.513[b] |
| Pulmonary disease | | 4 | 4.0 | 10 | 6.6 | 0.366[b] |
| Renal failure | | 0 | 0.0 | 1 | 0.7 | 1.000[b] |
| **Admission to palliative care from** | | | | | | |
| House | | 43 | 42.6 | 80 | 53.0 | 0.000[b] |
| Other hospital | | 18 | 17.8 | 45 | 29.8 | |
| Intensive care unit | | 40 | 39.6 | 26 | 17.2 | |
| **Vacuum assisted closure** | (-) | 83 | 82.2 | 151 | 100.0 | 0.000[b] |
| | (+) | 18 | 17.8 | 0 | 0.0 | |
| **Discharge status** | | | | | | |
| To home | | 28 | 27.7 | 60 | 39.7 | 0.049[b] |
| To intensive care unit | | 40 | 39.6 | 29 | 19.2 | |
| Transferred | | 27 | 26.7 | 48 | 31.8 | |
| Died | | 5 | 5.0 | 14 | 9.3 | |
| Nursing home | | 1 | 1.0 | 0 | 0.0 | |

[a] Mann-Whitney U test / [b] Chi-square test (Fischer test)

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The patients' age, gender, from where they were admitted, diagnosis on admission, comorbidities, length of hospitalization, discharge status, Braden scores in admission, oral intake insufficiency, the status of pressure ulcers, the pressure ulcer treatment method and the hospitalization costs of the patients were evaluated comparatively. In the study, prevalence means all patients who had pressure ulcers in admission and added new ones during hospitalization. Incidence means the patients who develop new pressure ulcers during the palliative care period.

## Statistical Analyses

Mean, standard deviation, median, minimum, maximum, frequency, and ratio values were used for the descriptive statistics of data. The distribution of variables was calculated by the Kolmogorov-Smirnov test. Independent quantitative data were analyzed by the Mann-Whitney U test. The Chi-Square test was used to examine independent qualitative data, and the Fischer test was used when the Chi-square test conditions were not met. SPSS 28.0 program was used in the analysis.

**Table 4.** Comparative analysis of patients whose pressure ulcers healed and those not

| | | Pressure ulcer | | | | |
|---|---|---|---|---|---|---|
| | | Healed | | Not healed | | |
| | | Mean ± SD | Median | Mean ± SD | Median | p |
| **Age (Year)** | | 71.7 ± 15.8 | 74.0 | 69.1 ± 17.4 | 72.0 | 0.571[a] |
| **Braden score** | | 11.3 ± 1.4 | 11.0 | 11.2 ± 2.0 | 11.0 | 0.812[a] |
| **Length of stay** | | 34.8 ± 39.2 | 23.5 | 55.7 ± 61.0 | 29.0 | 0.114[a] |
| **Cost of hospitalization (dollar)** | | 9112.1 ± 7396.1 | 6285.8 | 10867.0 ± 14405.7 | 4530.2 | 0.506[a] |
| | | **N** | **%** | **N** | **%** | **p** |
| **Sex** | Male | 11 | 55.0 | 42 | 51.9 | 0.801[b] |
| | Female | 9 | 45.0 | 39 | 48.1 | |
| **Comorbidity** | | | | | | |
| Diabetes mellitus | | 4 | 20.0 | 13 | 16.0 | 0.672[b] |
| Hypertension | | 6 | 30.0 | 16 | 19.8 | 0.320[b] |
| Heart disease | | 3 | 15.0 | 7 | 8.6 | 0.410[b] |
| Pulmonary disease | | 1 | 5.0 | 3 | 3.7 | 1.000[b] |
| **Admission to palliative care from** | | | | | | |
| House | | 8 | 40.0 | 35 | 43.2 | 0.948[b] |
| An other hospital | | 4 | 20.0 | 14 | 17.3 | |
| Intensive care unit | | 8 | 40.0 | 32 | 39.5 | |
| **Vacuum assisted closure** | (-) | 17 | 85.0 | 66 | 81.5 | 0.713[b] |
| | (+) | 3 | 15.0 | 15 | 18.5 | |
| **Discharge status** | | | | | | |
| To home | | 4 | 20.0 | 24 | 29.6 | 0.389[b] |
| To intensive care unit | | 11 | 55.0 | 29 | 35.8 | |
| Transferred | | 5 | 25.0 | 22 | 27.2 | |
| Died | | 0 | 0.0 | 5 | 6.2 | |
| Nursing home | | 0 | 0.0 | 1 | 1.2 | |

[a] Mann-Whitney U test / [b] Chi-square test (Fischer test)

## RESULTS

The data of 252 patients who received inpatient treatment in the palliative care unit between January 2016-2022 were analyzed. There were 137 (54.4%) male and 115 (45.6%) female patients. The mean age was 67.4±16.7. The most common comorbid diseases of the patients were hypertension (17.5%) and diabetes mellitus (13.5%). Almost half, 48.8%, of the patients were admitted from home. Mainly oncology (30.6%) patients and cerebrovascular disease (30.6%) patients were admitted (Table 1).

The Braden score of the patients was found to be a median of 13 (5-23). Pressure ulcers prevalence in our palliative care unit was 40.1%. Although daily wound care was performed in 81 (80.2%) patients with pressure ulcers, healing could not be achieved. It was observed that new pressure ulcers were added in 12 of the patients whose pressure ulcers did not heal. Pressure ulcers incidence in our palliative care unit was 4.7%. There was more than one pressure ulcer at admission in 49 (48.5%) patients with pressure ulcers. Vacuum-assisted closure (VAC) was used in the treatment of 18 patients with pressure ulcers. While 34.9% of the patients were discharged home, 29.8% were transferred to a tertiary-level center, and 27.4% were transferred to the intensive care unit. Nineteen (7.5%) patients died in the palliative care unit. The mean length of stay was 39.7 ± 51.6 days, and the mean cost of hospitalization was 8656.6 ± 13889.4 dollars (Table 2).

The Braden score and the rate of discharge to home were significantly lower in patients with pressure ulcers than those without wounds ($p<0.05$). The rate of admission from another intensive care unit, the length of hospital stay, and hospitalization cost was significantly higher in the group with pressure ulcers than in the group without pressure ulcers ($p<0.05$) (Table 3).

In the comparative analysis of patients whose pressure ulcers healed and those who did not, no significant difference was found in terms of where they were admitted from, Braden scores, rate of vacuum assisted closure (VAC) use, the rate of discharge home, the length of hospital stay, and hospitalization cost ($p>0.05$) (Table 4).

In the comparative analysis of patients whose pressure ulcers worsened and whose were stable, it was observed that the rate of VAC use and the cost of hospitalization were significantly higher in patients with worsening pressure ulcers ($p<0.05$) (Table 5).

## DISCUSSION

The increasing elderly population, chronic diseases, and cancer have increased the need for palliative care. Palliative care aims to feel the patients and their relatives well in the last period of life, and it has been fully integrated into healthcare systems in developed countries. In modern healthcare systems, every patient has the right to palliative care (8).

With aging, there are changes such as thinning of the subcutaneous tissue, loss of collagen and elastic fibers, decrease in vascularity, and disruption of the skin's barrier function. Additional factors such as a deterioration in nutritional status, cachexia, and immunosuppression due to cytokines such as tumor necrosis factor (TNF) α, as in cancer patients, or accompanied by immobilization and loss of sensorimotor sensation, as in cerebrovascular disease, further increases pressure ulcer development risk. In addition, it should be remembered that in the last period of life, inevitable pressure ulcers may be encountered due to the inadequacy of homeostatic mechanisms. For all these reasons, palliative care patients are candidates for pressure ulcer development (9).

Pressure ulcers prevalence in palliative care is between 17% to 47% in the literature (10). In Galvin's study, pressure ulcers prevalence in the palliative care unit was 38.1%, and the incidence was 12% (11). Hendrichova et al. reported the pressure ulcers prevalence as 22.9% and the incidence as 6.7% in cancer patients in the palliative care unit (10). When we look at the data from Turkiye, Gencer et al. found the general prevalence of pressure ulcers in a university hospital was 2.49% and the incidence 1.9% (12). Studies conducted in the intensive care unit show that the rate of pressure ulcers varies between 15-29% (13). In their study, Zengin and Tasci found that the pressure ulcer frequency in the palliative care unit is as high as 36.1% (1). In our study, pressure ulcers prevalence was 40.1%, and the incidence was 4.7%. This result shows that if correct risk analysis is performed and protection is taken, the incidence of pressure ulcers could decrease even if the prevalence is very high.

In our study, the mean age, gender distribution, diagnoses in admission, and comorbid diseases of the patients were compatible with the literature.

Since the frequency of pressure ulcers varies from each other in the general population, clinical units, intensive care units, and palliative care units, all patients should be well questioned about where they are admitted from and their previous care history. The risk of pressure ulcers is higher in patients with a history of organ dysfunction that requires intensive care follow-up. Our study observed that admission from the intensive care unit was significantly higher in patients with pressure ulcers.

The Norton, Braden, and Waterlow scales are commonly used scales for pressure ulcer risk assessment of patients (14). Pressure ulcer risk analysis is performed by evaluating sensory perception, activity, mobility, humidity, nutrition, and friction/shearing with the Braden scale, and patients receive a score between 6 and 23. The risk of pressure ulcers increases as the Braden scale total score decreases (7). Our study's median Braden score was 13 (5-23). This low score shows the high risk of pressure ulcers in palliative care patients. Additionally, the Braden score was significantly lower in patients with pressure ulcers than those without wounds. This result indicates we can safely use the Braden scale in palliative care unit patients.

The rate of discharge to home in a palliative care unit is accepted as one of the indicators of success (1). Yuruyen et al. found the discharge rate to home in the tertiary level palliative care unit to be 52%. In the same study, the transfer rate to intensive care was 15.3%, and mortality in the palliative care unit was 33.4% (15). Starks et al. in their study, which used data from two different hospitals' palliative care units, found the discharge rate to home to be 13.4% and 15.3%, respectively. In the same study, the rate of discharge to a skilled nursing facility was 15.3% and 33.0%, and the rate of discharge to a hospice was 22.1% and 3.3%. Starks et al. found 37.3% and 39.5% mortality rates in the palliative care unit in their study (16). In our study, 34.9% of the patients were discharged home, while 29.8% were transferred to a tertiary level center and 27.4% to the intensive care unit. The mortality rate was found to be 7.5%. Our study observed that the discharge rate to home was significantly

**Table 5.** Comparative analysis of patients whose pressure ulcers worsened and those stable

| | | Pressure ulcer | | | | p |
|---|---|---|---|---|---|---|
| | | Worsened | | Stable | | |
| | | Mean ± SD | Median | Mean ± SD | Median | |
| **Age (Year)** | | 67.1 ± 17.3 | 70.0 | 69.8 ± 17.5 | 72.0 | 0.595[a] |
| **Braden score** | | 10.7 ± 1.5 | | 11.4 ± 2.1 | | 0.153[a] |
| **Length of stay** | | 62.8 ± 55.1 | | 53.3 ± 63.0 | | 0.369[a] |
| **Cost of hospitalization(dollar)** | | 11638.0 ± 7927.9 | | 10614.3 ± 16016.7 | | 0.033[a] |
| | | **N** | **%** | **N** | **%** | **p** |
| **Sex** | Male | 10 | 50.0 | 32 | 52.5 | 0.849[b] |
| | Female | 10 | 50.0 | 29 | 47.5 | |
| **Comorbidity** | | | | | | |
| Diabetes mellitus | | 3 | 15.0 | 10 | 16.3 | 0.224[b] |
| Hypertension | | 3 | 15.0 | 13 | 21.3 | 0.538[b] |
| Heart disease | | 2 | 10.0 | 5 | 8.2 | 1.000[b] |
| Pulmonary disease | | 0 | 0.0 | 3 | 3.7 | 0.571[b] |
| **Admission to palliative care from** | | | | | | |
| House | | 5 | 25.0 | 30 | 49.2 | 0.158[b] |
| An other hospital | | 5 | 25.0 | 9 | 14.8 | |
| Intensive care unit | | 10 | 50.0 | 22 | 36.1 | |
| **Vacuum assisted closure** | (-) | 13 | 65.0 | 53 | 86.9 | 0.029[b] |
| | (+) | 7 | 35.0 | 8 | 13.1 | |
| **Discharge status** | | | | | | |
| To home | | 3 | 15.0 | 21 | 34.4 | 0.099[b] |
| To intensive care unit | | 13 | 65.0 | 16 | 26.2 | |
| Transferred | | 3 | 15.0 | 19 | 31.1 | |
| Died | | 1 | 5.0 | 4 | 6.6 | |
| Nursing home | | 0 | 0.0 | 1 | 1.6 | |

[a] Mann-Whitney U test / [b] Chi-square test (Fischer test)

lower in patients with pressure ulcers than in those without. This result shows that the development of pressure ulcers directly affects the success of palliative care.

Although the rate of discharge to home was consistent with the literature in our study, transfer rates to a tertiary-level center and intensive care unit were found to be high since our unit is located in a secondary-level hospital. The 7.5% mortality rate for the palliative care unit seems relatively low. The mortality rate was low because the discharge status of the patients transferred to a tertiary-level center and intensive care unit was unknown and was not evaluated in this study.

Unfortunately, the capacity of palliative care units, which has yet to be fully integrated into healthcare systems in many countries, to afford the supply-demand balance is also limited. Therefore, how long it takes to discharge home successfully is extremely important for both patient and their relatives, and the burden on the healthcare system. Factors such as a limitation of movement, feeding tube, permanent tracheostomy, hypertension, infection, and pressure ulcers affect the duration of stay in the palliative care unit (1). Our study observed that the length of stay in the palliative care unit was longer in patients with pressure ulcers than in patients without ulcers.

Cost analyses are essential to understand the burden of palliative care units on the healthcare system and to produce appropriate solutions. Pressure ulcers increase the length of hospital stay and treatment costs and the burden on the healthcare system. Our study observed that the financial burden of patients with pressure ulcers on the healthcare system was significantly higher.

Comparative analyses of patients whose pressure ulcers healed and those who did not, revealed that no real success can be achieved even if the best care services and treatment options are offered after pressure ulcers develop in the palliative care unit. The length of stay in the palliative care unit of the patients whose wounds healed was as long as those who did not. Similarly, the discharge rate to home was found to be low, and the costs increased. This result shows the importance of pressure ulcer risk analysis and prevention of pressure ulcers before they develop.

Comparative analyses of patients with worsening pressure ulcers and patients with stable pressure ulcers showed no significant difference in the rate of discharge to home and length of hospital stay between the two groups and that the use of VAC and costs were higher in patients with worsening pressure ulcers. This result shows that despite the expensive treatment such as VAC, real success has yet to be achieved, and the costs have increased (Table 5).

The most important limitations of our study were that it is retrospective and with a limited number of patients. In addition, since our palliative care unit is in a secondary level health care institution, the final status of the patients transferred to a tertiary-level center could not be evaluated.

In conclusion, pressure ulcers negatively affect the patient's length of stay in the palliative care unit, the rate of discharge to home, and the financial burden on the healthcare system. In order to obtain cost-effective and successful results in palliative care units, a pressure ulcer risk analysis and a standardized care plan to prevent and treat pressure ulcers should be performed.

**Ethics Committee Approval:** The study's ethical approval was obtained by the local ethics committee of Haseki Training and Research Hospital (Approval number: 71-2022).

**Conflict of Interest:** The author declare that there is no conflict of interest.

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

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Experimed 2023; 13(2): 142-147

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1246321

# Investigation of the Effect of Serum IL-1β Levels on Atherosclerosis: A Turkish Population-Based Study

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**Cite this article as:** Dlawer Omer D, Akdeniz FT, Gulec Yilmaz S, Barut Z, Billur D, Turer Cabbar A, Isbir T. Investigation of the effect of serum IL-1β levels on atherosclerosis: A Turkish population-based study. Experimed 2023; 13(2): 142-147.

## ABSTRACT

**Objective:** Atherosclerosis, as a cardiovascular disease associated with chronic inflammatory conditions and involving a large number of risk factors, harbors a lot of unknowns. Evidence has emerged that almost all stages of the disease are intertwined with inflammation. It has not been fully elucidated in which stages and how interleukin-1 beta (IL-1β), which is associated with inflammations, plays a role in the chronic inflammatory process of the disease. Also, the effect on atherosclerosis progression and biomarker candidacy of IL-1β in the Turkish population is unknown. The aim of this study was to investigate the serum IL-1β expression levels and whether it is associated with changes in risk factors that develop due to the disease in the Turkish population.

**Materials and Methods:** ELISA method was used to analyze the serum IL-1β levels. Statistical data was obtained utilizing the SPSS v.23 software.

**Results:** No linkage evidence was found between the serum IL-1β and atherosclerosis ($p>0.05$). However, the serum IL-1β levels were significantly associated with gender, smoking status, and diabetes history within the study population were significantly associated ($p=0.050$). According to the lipid profile result, the triglyceride value was found to be significantly decreased in atherosclerosis patients when compared to healthy individuals ($p=0.012$). Additionally, factors including, gender, smoking status, and history of diabetes were significantly associated with serum cholesterol level ($p=0.015$).

**Conclusion:** Although no correlation was found between the serum IL-1β levels of the study groups, it was observed that the serum IL-1β expression alters in patients with atherosclerosis depending on age. In addition, low triglyceride levels may be a marker of disease in atherosclerosis patients. Studies involving different ethnic groups with a large sample and identifying the stages of the disease are necessary to confirm these results.

**Keywords:** Atherosclerosis, inflammation, IL-1β, triglyceride, lipid level, serum

## INTRODUCTION

Atherosclerosis, which is considered to be one of the leading diseases among the causes of death worldwide, is a multi-stage disease in which many factors are involved (1). Due to the age-related development of atherosclerosis and the effect of inflammation on the formation of the disease, it has become clear through recent studies that age and inflammation can be counted among the risk factors of atherosclerosis (2, 3). There is increasing evidence that aging has not been associated with atherosclerosis as a one-way relationship (2). The changes that occur in the vessels, especially with increased age, are similar to several characteristic pathological processes

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**Submitted:** 01.02.2023 **Revision Requested:** 13.03.2023 **Last Revision Received:** 27.03.2023 **Accepted:** 24.04.2023 **Published Online:** 10.07.2023

seen in atherosclerosis presentations (4). It is reported that proinflammatory changes in monocytes/macrophages in the development of atherosclerosis change with age. One of the most important factors underlying the molecular infrastructure of atherosclerosis is the alteration in the expression of cytokines. An increase in the level of free radicals was parallelly observed with the activation and increased secretion of many cytokines, including interleukin-1 beta (IL-1β), within obtained data from patients with atherosclerosis (5).

The pathways that trigger or participate in inflammation determine the direction of atherogenesis, so risk factors linked with these pathways have long been associated with each stage of atherosclerosis and its complications. Although many molecular actors like interleukins (ILs) are detected in cardiovascular diseases, IL-1β has emerged as a therapeutic target among these ILs (6). A substantial number of studies have demonstrated the effect of IL-1β on atherosclerosis along with varying properties at different stages (7-9). The inflammatory response that develops in endothelial cells, which is known to occur especially in the primary stage of atherosclerosis, and the accumulation of inflammatory factors induced by this response in the vessels are promoted by IL-1β (6). IL-1β has a pivotal effect on atheroma growth. It is associated with a lower inflammatory response, increased IL-10 expression, and reduced plaque size when neuralized in studies on atherosclerosis disease (10). IL-1β, a member of the IL-1 family associated with coronary diseases, is significantly elevated in both mRNA and protein levels in patients with atherosclerosis as shown in various case-control studies (11, 12). The contribution of IL-1β in the development and progression of the disease is also related to the signals that cause its expression and the molecules involved in its synthesis. IL-1β is a product largely produced by macrophages and monocytes as part of a multi-step process. Activation of the precursor IL-1β is triggered by the accumulation of molecules such as cholesterol crystals and oxidized low-density lipoprotein (LDL). The maturation process is carried out by caspase-1 or various enzymes detected in human atherosclerotic plaques (6). Therefore, changes in the lipid profile are key factors in the course of the disease by contributing to the formation of a pro-inflammatory response in the development of atherosclerosis, and are important markers for understanding the molecular mechanism of this disease (6). In addition to cholesterol crystals and oxidized LDL, triglycerides are associated with the development of atherosclerosis. It has been reported that a high triglyceride level is a risk factor for coronary heart disease (13).

Since atherosclerosis is a disease that threatens public health worldwide, like other cardiovascular diseases, its exact molecular mechanism unknown, the expression level of IL-1β as a cytokine, aging, and lipid profiles that are reported to play important roles in the course of this disease have been investigated in the Turkish population.

## MATERIALS AND METHODS

### Study Population and Clinical Procedures

This study was performed per tenets of the Declaration of Helsinki. Ethical approval was obtained for this study from the Yeditepe University Medical Faculty Ethics Committee (Date: 01/30/2020 and No: 1793). All participants were selected and examined by the Department of Cardiology, Yeditepe University Hospital, and signed an informed consent form prior to the investigation. A total of 69 individuals of which thirty-nine were patients (31 males / 8 females) diagnosed with atherosclerosis were utilized. The control group involved thirty healthy subjects who do not have any disease including cardiovascular diseases (26 males / 4 females). Age ranges of subjects were between 30 and 80 years. Parameters such as age, gender, smoking status, diabetes history, and serum lipid levels (high-density lipoprotein (HDL), LDL, and triglyceride) of the study population were obtained from the hospital.

### IL-1β Determination by ELISA

Following ethical procedures, the determination of serum IL-1β levels was performed using the commercial Human IL-1β Enzyme Linked Immunosorbent Assay (ELISA) assay kits (Biosource, Nivelles, Belgium), according to the manufacturer's instructions, and using the microplate reader with the ability to measure absorbance at 450 nm (WHYM201). The detection limit was 0.06 pg/ml for IL-1β.

### Statistical Analysis

Statistical analysis was performed using the SPSS-22 software (SPSS, Inc, Chicago, IL, USA), and the values were expressed as mean ± standard deviation (SD). The value's significance level was $p < 0.05$. The demographic records were compared and the serum IL-1β levels of the study population were analyzed by the student's t-test, Chi-square test, one-way ANOVA, and the Mann-Whitney U test.

## RESULTS

Table 1 shows the demographic characteristics of the study participants. Of the sixty-nine total individuals, thirty-nine were patients (79.0% male, 21.0% female) with ages of 62.95 ± 10.96 years, and 30 individuals were healthy controls (87.0% male, 13.0% female) aged 59.96 ± 9.36 years. The mean age ($p=0.143$) and gender ($p=0.533$) of the study population were not significantly different. In 33.0% of patients and 17.0% of healthy individuals, a history of diabetes was present. There was no significant difference between the study groups regarding this parameter ($p=0.698$). While the frequency of smoking was 38.0% among those with atherosclerosis, it was 53.0% in the group of healthy individuals. As shown in Table 1 smoking data of both groups was not statistically significant ($p=0.470$).

When the effect of serum lipid levels on disease progression in human serum samples with atherosclerosis and healthy

**Table 1.** Demographic data of the study subjects.

| Characteristic | Patient (n=39) | Control (n=30) | p-value |
|---|---|---|---|
| **Age (Years; mean± SD)** | 62.9 5± 10.96 | 59.96 ± 9.36 | 0.143 |
| **Gender (Male / Female; %)** | 79.0 / 21.0 (n=31) / (n=8) | 87.0 / 13.0 (n=26) / (n=4) | 0.533 |
| **History of Diabetes (Yes / No; %)** | 33.0 / 67.0 (n=13) / (n=26) | 17.0 / 83.0 (n=5) / (n=25) | 0.698 |
| **Smoking (Yes / No; %)** | 38.0 / 62.0 (n=15) / (n=24) | 53.0 / 47.0 (n=16) / (n=14) | 0.470 |

n=number of the sample; SD: Standard deviation

**Table 2.** Comparison of total cholesterol, triglycerides, LDL, HDL parameters of the study population.

| Parameter | Patient (n=39) | Control (n=30) | p-value |
|---|---|---|---|
| **Cholesterol (mg/dl)** | 185.15 ± 7.97 | 174.40 ± 6.25 | 0.34 |
| **Triglycerides (mg/dl)** | 132.31 ± 6.68 | 142.33 ± 14.63 | 0.012* |
| **LDL (mg/dl)** | 120.61 ± 6.72 | 105.23 ± 6.19 | 0.329 |
| **HDL (mg/dl)** | 36.74 ± 1.14 | 40.23 ± 1.55 | 0.685 |

n=number of the sample; *p<0.05

individuals were examined, no relationship was found between cholesterol (p=0.34), LDL (p=0.329), and HDL (p=0.685) concentrations and atherosclerosis, except for triglycerides. In the group of patients with atherosclerosis, it was determined that the triglyceride level was significantly decreased (p=0.012) (Table 2). Although no difference was found in cholesterol levels between the patient and control groups in our study population, it was found that gender, smoking status, and diabetes history were associated with cholesterol levels (p=0.015).

Serum IL-1β levels were measured in both patient and control groups. Although lower IL-1β levels were detected in the patient group (12.90 ± 7.538 pg/ml) as compared to the control group (13.47 ± 19.565 pg/ml), no significance was found (p>0.05). Additionally, representative histograms of serum IL-1β and triglyceride concentrations are given in Figure 1 and 2.

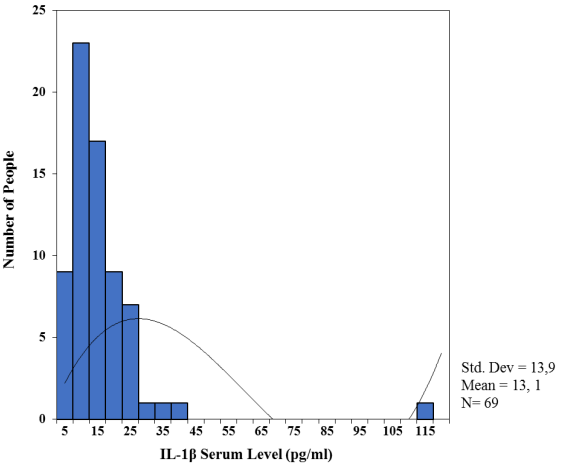


**Figure 1.** Representative histogram of IL-1β.

Further, to understand how the serum IL-1β level changes depending on age in both the patient and control groups, analyses were performed in three different age groups: less than 50 years old (<50), between 50-60 years old, and over 60 years old (60>). Out of thirty-nine patients with atherosclerosis, serum IL-1β expression levels at <50, 50-60, and 60> age groups were 17.23 ± 19.23 pg/mL, 10.93 ± 6.19 pg/mL, and 13.07 ± 6.47 pg/mL, respectively. Out of thirty healthy individuals, the IL-1β expression level results for <50, 50-60, and 60> age groups were as follows; 11.86 ± 6.28 pg/mL, 7.28 ± 5.27 pg/mL, and 19.11 ± 28.57 pg/mL. While the serum IL-1β level in the patient group was higher in the group aged 50 years and below, the opposite result was obtained in the control group. In the control group, the highest level of IL-1β was detected in the 60 years and older group (Figure 3).

Within the scope of our research, it was also investigated whether there was any association between the demographic and clinical data of the study groups and IL-1β expression level. Smoking and history of diabetes (p=0.143) and smoking and

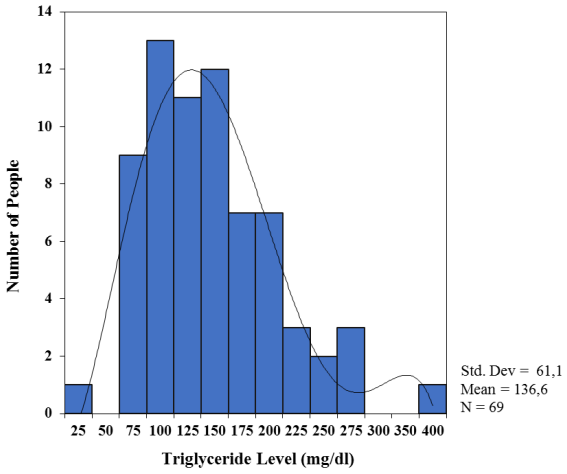


**Figure 2.** Representative histogram of serum triglyceride levels.

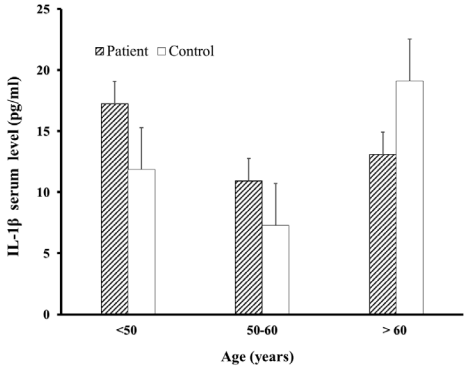


**Figure 3.** Distribution of serum IL-1β level in both patients with atherosclerosis and control groups by age.

gender (p=0.541) did not show any significant difference for IL-1β expression levels between the groups. Diabetes history alone was also not significantly related to serum IL-1β between the groups (p=0.833). On the other hand, serum IL-1β levels with gender, smoking, and diabetes history within the study population were significantly associated (p=0.050).

## DISCUSSION

In literature, this is the first study to investigate the IL-1β expression level in human serum samples, its age-related change in atherosclerosis, and the serum concentration of triglyceride value in patients with atherosclerosis in the Turkish population.

In humans, the role of the proinflammatory cytokine IL-1β in atherosclerosis is not well-defined. By designing a case-control study in the Turkish population that included individuals with atherosclerosis and those without atherosclerosis, we examined the association of clinical data with the disease and the expression profile of the serum inflammatory cytokine IL-1β. Extensively investigated IL-1β levels by researchers all over the world was not a risk factor for atherosclerosis when compared to healthy individuals in the Turkish population. In this study population it showed that IL-1β alone does not play a role in the disease and cannot be a stand-alone biomarker for atherosclerosis.

The great majority of studies were conducted utilizing animal experiments, and very few population-based human studies have been performed. In addition, studies with the IL-1 family generally examined the consequences of their blockade of disease development and progression (14, 15). The common consensus that emerged from studies that have accumulated over the years was that inflammation has a visible link in coronary artery disease progression and each stage of the atherosclerosis process (16).

An animal study by Vromman et al. provided evidence that blocking IL-1β mainly brought blood monocytes to a less inflammatory state during atherogenesis, reduced atheroma size, and increased plasma levels of IL-10 (10).

One of the few population-based studies in the literature, Di Iorio et al. within the scope of the InCHIANTI project, measured IL-1β levels in 1,292 human samples collected from two different cities in Italy. As a result of the study, it showed that IL-1β did not change depending on age and gender but was associated with clinical conditions such as angina and serum calcium level (16). A cross-sectional study was performed by Joung et al. to determine whether IL-1β could be a marker for cardiovascular disease (15). As in our study, no significant relationship was found between the patients and study groups regarding serum IL-1β levels. However, when its relationship with other parameters in the study was examined, it could be an independent risk factor for newly diagnosed cardiovascular disease in smoking patients (16). Though the link of IL-1β expression with atherosclerosis development and progression has been shown in these studies, no significant relationship has been found between IL-1β expression and atherosclerosis in this study. Such contradictory results may be because the studies were conducted on different ethnic groups, as well as the sample size, human serum samples that were used, the nutritional preferences of the individuals included in the study, and the drugs used by the individuals.

In the context of this study, we examined whether the IL-1β expression levels change with regards to age. In patients with atherosclerosis, the IL-β serum concentration was highest in the age group of 50 and below (17.23 ± 19.23 pg/mL). IL-1β concentration reached the highest value in the 60-year and older group within the control group (19.11 ± 28.57 pg/mL). Despite these results, there was not a significant difference within or between the groups. Studies showing that aging is an independent risk factor for atherosclerosis are growing exponentially. In addition to the changes in the aging vessels, alterations in the expression profiles of the proinflammatory molecules, IL-1β, were observed (17). The most plausible explanation for the higher concentration of IL-1β expression in atherosclerosis patients under 50 years of age in our study

population may be that this cytokine is activated in the early stages of inflammation (18). In the control group, the high concentration measured in individuals aged 60 and older can be considered as sign of age-related inflammatory processes.

Parallel to the increased expression of proinflammatory cytokines, there was an increase in the level of atherogenic lipoprotein. Therefore, the lipid profiles of the study groups were measured, and their association with atherosclerosis was assessed. This study reported that triglyceride levels significantly decreased in atherosclerosis patients (p=0.012). Although many studies were conducted regarding high triglyceride levels and increased IL-1β activity in relation to the disease, few studies reported on the contribution of low triglyceride to the biology of atherosclerosis.

Although decreased serum triglyceride levels are predictors of cardiovascular death in patients with heart failure, the reasons for the low triglyceride levels remain unknown and require further investigation. This decline may be explained by various mechanisms. Studies that revealed these possibilities are available in the literature.

Research on chronic heart failure reported a link between low triglyceride levels and the disease. It showed that decreased triglyceride levels could be an independent marker of cardiac death (19). In addition, factors such as nutritional deficiency and increased inflammation could also be among the causes of changes in triglyceride levels. Since the triglyceride value of 150mg/dl is accepted as an indicator that does not pose a risk to human health, it is important to understand the atherosclerosis of patients in our selected population with an average of 132.31mg/dl level (20). Also, the decreased level of IL-1β (albeit non-significant) as an outcome of this study may have contributed to the low serum triglyceride levels in atherosclerosis by preventing lipolysis. This kind of change in cytokine activity can alter the molecular response to inflammation by affecting the serum triglyceride level, leading to a change in the lipid profile.

## Study Limitations

This study contained some limitations that should be discussed. The most important of these is that the sample size was not large enough to achieve substantial results. In addition, the fact that only a single population was studied could have caused the results to differ from previous studies. The varied ages of the individuals in the study groups was another factor. Different age groups may cause different outcomes, so it may be necessary to narrow the age range in future studies. In addition, there is a close relationship between cardiovascular diseases and nutrition, therefore, the dominance of the Mediterranean diet in the Turkish population may have also affected the results.

## CONCLUSION

Consequently, although a significant relationship could not be demonstrated, this study exhibited data showing that monitoring and analyzing serum IL-1β levels according to age groups could provide information regarding the course of atherosclerosis. In addition, this study is one of the few examining the relationship between serum IL-1β concentration and atherosclerosis amidst the Turkish population. It was found that the triglyceride level decreased in this patient group population. These results did not support the role of decreased IL-1β level in atherosclerosis in the Turkish population, but consequently showed that triglyceride levels decreased in patients with atherosclerosis. It will be important to carry out further studies utilizing larger sample groups and different ethnic populations regarding the possible elements of the molecular mechanism that caused this result.

**Ethical Approval:** All procedures performed in studies involving human participants were under the ethical standards of the 1975 Declaration of Helsinki guidelines and its later amendments. The research on humans study protocol was approved by the Yeditepe University Medical Faculty Ethics Committee (Date: 01/30/2020 and No: 1793).

**Authors' Contributions:** Conception/Design of Study – T.I., D.D.O.; Supervision – T.I., Data Acquisition – A.T.C.; Performing experiments – D.D.O., F.T.A.; Analysis and/or Interpretation – S.G.Y., F.T.A., D.D.O., Z.B., D.B.; Drafting Manuscript – T.I., D.D.O., D.B.; Critical Revision of Manuscript – T.I., S.G.Y., F.T.A., D.D.O., Z.B., A.T.C., D.B.; Final Approval and Accountability– T.I. All authors read, revised, and approved the final article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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

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Experimed 2023; 13(2): 148-155

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1308286

# The Potential Role of Hematological and Biochemical Parameters in Pregnant Women with Viral Infection

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**Cite this article as:** Ergun NE, Surmen S, Erturk S, Buyukkayhan D, Surmen MG. The potential role of hematological and biochemical parameters in pregnant women with viral infection. Experimed 2023; 13(2): 148-155.

## ABSTRACT

**Objective:** Pregnancy is a process in which not only physical changes occur, but also immune responses are modulated. Therefore, pregnant women are more susceptible to viral infections due to these changes during the pregnancy. The present study aimed to evaluate the hematological and biochemical parameters of pregnant women diagnosed with viral infection.

**Materials and Methods:** The study groups consisted of pregnant women diagnosed with cytomegalovirus (CMV), hepatitis B virus (HBV), influenza, Covid-19 and healthy pregnant women as control group. The data of a total of 522 pregnant women were analyzed retrospectively. SPSS 22.0 statistics package program was used for data analysis.

**Results:** Significant differences were found in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, and neutrophil counts between CMV and control groups. There were also significant differences in AST, ALT, red blood cell distribution width (RDW), neutrophil lymphocyte ratio (NLR), white blood cell (WBC), platelet (PLT), neutrophil and lymphocyte values between hepatitis B surface antigen (HBsAg) positive and HBsAg negative pregnant groups. NLR and basophil (BASO) % of pregnant women with influenza infection were significantly higher than the control group. In Covid-19 positive pregnant women, AST, ALT, WBC, BASO values and BASO% were found to be significantly different from the control group. In addition, WBC and NLR values in Covid-19 positive pregnant women were found to be significantly lower than the influenza group.

**Conclusion:** All these results suggest that especially the NLR value may have diagnostic and prognostic significance for viral infections in pregnant women. However, studies with larger samples and prospective analyses are needed for stronger evidence.

**Keywords:** Viral infection, neutrophil lymphocyte ratio, pregnancy, retrospective

## INTRODUCTION

Pregnant women are at high risk for viral infections and related complications due to the physiological and immunological changes during pregnancy. The adaptation of the immune system to tolerate the allogeneic fetus in this process is among the main reasons for these changes (1).

Cytomegalovirus (CMV) is one of the common congenital contagious infections. Serological screening test is performed during pregnancy for the diagnosis of primary infection of CMV. However, detection of DNA for CMV by amniocentesis is considered as the gold standard for diagnosis in the estimation of primary infection. CMV infection can cause serious sequelae such as neurological

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**Submitted:** 01.06.2023 **Revision Requested:** 11.07.2023 **Last Revision Received:** 19.07.2023 **Accepted:** 29.07.2023 **Published Online:** 04.08.2023

disability, hearing loss and vision loss in new-borns. Although the primary infection during pregnancy is asymptomatic in most of the mothers, care should be taken because of the sequelae that may occur later in the newborn (2).

Another issue that may pose a serious risk for both mother and fetus during pregnancy is hepatitis B virus (HBV) infection. HBV infection carries serious risks for the mother, especially in the second trimester and during delivery. In addition, pregnant women with high viremia are at risk of mother-to-child transmission of HBV (3). For this reason, follow-up during pregnancy and postpartum against pregnancy-associated hepatitis B reactivation is of great importance.

On the other hand, the literature contains various information regarding the influenza virus infection, which caused pandemics in the past due to its rapid spread potential, affecting both maternal health and pregnancy outcome. The study results implied that disease severity was associated with preterm birth and stillbirth. The fact that pregnant women are more susceptible to flu-related complications has been attributed to immunological (changes in T and B cell counts) and physiological adaptations (increased respiratory rate and intra-abdominal pressure) during pregnancy (4).

Nowadays, the Covid-19 pandemic, which affected millions of people, was experienced. The main symptoms of Covid-19 such as fever, cough and shortness of breath have clinical manifestations similar to influenza (5). Some hematological parameters have been reported and can serve as diagnostic and prognostic markers depending on Covid-19 severity (6).

In the fight against viral infections, including influenza and Covid-19, anti-viral treatments and vaccinations can help to prevent negative pregnancy outcomes. However, information on the adverse effects of vaccines and antiviral treatments on mother and baby is limited. In this context, it is critical to detect viral infections before they pose a threat to maternal and infant health.

This study aimed to evaluate the relationship between viral infections during pregnancy and pregnant women's hematological and biochemical parameters.

## MATERIALS AND METHODS

Istanbul Medeniyet University Goztepe Training and Research Hospital, Clinical Research Ethics Committee approval was obtained for this study (Date: 10.02.2021, Decision No: 2021/0140).

### Data Collection

The present retrospective study aimed to evaluate the hematological and biochemical parameters of pregnant patients diagnosed with viral infection. Data were collected from 555 pregnant patients and 141 healthy pregnant women who attended to the Istanbul Esenler Obstetrics and Pediatrics Hospital and Istanbul Haseki Sultangazi Training and Research Hospital, Gynecology outpatient clinic, between the years 2018 and 2020. Among these, 140 pregnant women with HBV infection, 4 pregnant women with Covid-19 infection and 30 pregnant women with CMV infection were subsequently excluded due to missing data. Pregnant women with gestational diabetes mellitus (GDM), preeclampsia, hypothyroidism, hyperthyroidism, pregnancy-related autoimmune diseases, and different concomitant viral infections were also excluded.

Patients' symptoms at presentation, white blood cell (WBC, $10^3$/µL) count, red blood cell (RBC, $10^{12}$/L) count, platelet (PLT, $10^3$/µL) count, mean platelet volume (MPV, fL), platelet distribution width (PDW, %), neutrophil (NEU, $10^3$/µL) count and percent, basophil (BASO, $10^3$/µL) count and percent, eosinophil (EOS, $10^3$/µL) count and percent, monocyte (MONO, $10^3$/µL) count and percent, lymphocyte (LYM, $10^3$/µL) count, red blood cell distribution width (RDW, %), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L) and neutrophil lymphocyte ratio (NLR) were evaluated. Routine blood tests were performed using the fluorescence flow cytometry (FFC) method and an automated hematology analyzer (Sysmex XN-1000). Using an automated biochemical analyser, AST and ALT parameters were measured by the enzymatic colorimetric method, and vitamin D (ng/mL) was determined by paramagnetic particle chemiluminescent immunoassay (Beckman Coulter AU680). Anti-CMV IgG and HBsAg were analyzed by chemiluminescent microparticle immunoassay (CMIA) (Architect i2000SR and Architect i1000, Abbott).

### Statistical Analyses

The IBM SPSS Statistics 22 program was used for statistical analysis of this study. The distribution of variables was evaluated with Kolmogorov-Smirnov and Shapiro Wilks tests. Student's t test was used to compare normally distributed variables, and the Mann-Whitney U test was used to compare non-normally distributed continuous variables. The correlation between the NLR value and gestational week were determined by the Spearman's correlation test. The Pearson's correlation analysis was also used to evaluate the relationship between vitamin D and ALT levels. Statistical significance was taken as p-values less than 0.05.

## RESULTS

### CMV Infection

The first group consisted of 53 CMV positive and 141 CMV negative pregnant women. The mean age of the CMV and control groups was 28.6 ± 5.2 and 29.2 ± 4.3, respectively ($p>0.05$). In this study group, WBC, NEU, LYM, AST and ALT values, which are routine blood parameters of pregnant women, were evaluated retrospectively. AST and ALT values of the pregnant group with CMV infection were found to be statistically significantly higher than the CMV negative control group. While no significant difference was observed in WBC and LYM values, the NEU value was found to be significantly lower than the control group. The other parameters are presented Table 1.

**Table 1.** Comparison of CMV and control groups.

| | CMV (n=53) | Control (n=141) | p-value |
|---|---|---|---|
| AST | 29.68 ± 24.92 | 18.36 ± 32.9 | [2]0.001* |
| ALT | 14.16 ± 3.75 | 12.22 ± 18.56 | [2]0.000* |
| WBC | 10.16 ± 4.04 | 10.14 ± 3.09 | [2]0.744 |
| NEU | 67.06 ± 8.51 | 70.16 ± 13.59 | [2]0.019* |
| LYM | 21.68 ± 8.37 | 20.45 ± 7.13 | [1]0.463 |

Cytomegalovirus (CMV), aminotransferase (AST), alanine aminotransferase (ALT), white blood cell (WBC), neutrophil (NEU), lymphocyte (LYM), [1]Student t Test, [2]Mann Whitney U Test, all values are shown as mean ± SD, SD: Standard deviation, *p<0.05

**Table 2.** Comparison of HBsAg positive and HBsAg negative groups.

| | HBsAg (+) (n=135) | HBsAg (-) (n=66) | p-value |
|---|---|---|---|
| MPV | 9.72 ± 0.86 | 9.78 ± 1.41 | [1]0.715 |
| AST | 24.25 ± 7.27 | 16.21 ± 4.95 | [1]0.000* |
| ALT | 15.6 ± 9.89 | 13.46 ± 8.2 | [2]0.007* |
| RDW | 13.6 ± 3.98 | 14.17 ± 1.42 | [2]0.000* |
| PDW | 10.69 ± 1.56 | 16.14 ± 1.57 | [1]0.000* |
| NLR | 1.59 ± 1.39 | 3.55 ± 1.42 | [1]0.000* |
| Vitamin D | 16.49 ± 6.56 | 19.44 ± 9.14 | [1]0.194 |
| WBC | 8.48 ± 2.13 | 9.51 ± 2.43 | [2]0.003* |
| PLT | 306.63 ± 75.59 | 246.34 ± 83.38 | [1]0.000* |
| NEU | 4.67 ± 4.18 | 6.70 ± 2.34 | [2]0.000* |
| LYM | 3.19 ± 1.02 | 2.03 ± 0.57 | [2]0.000* |

Mean platelet volume (MPV), aminotransferase (AST), alanine aminotransferase (ALT), red blood cell distribution width (RDW), platelet distribution width (PDW), neutrophil lymphocyte ratio (NLR), white blood cell (WBC), platelet (PLT), neutrophil (NEU), lymphocyte (LYM), [1]Student t Test, [2]Mann Whitney U Test, all values are shown as mean± SD, SD: Standard deviation, *p<0.05

## Hepatitis B Surface Antigen (HBsAg) Infection

Group 2 consisted of 135 HBsAg positive and 66 HBsAg negative pregnant women. The mean age of the influenza and control groups was 27.2 ± 4.7 and 26.3 ± 4.9, respectively (p>0.05). The NLR was calculated from these parameters. While the WBC and NEU values of the HBsAg positive pregnant group were statistically significantly lower than the healthy control group, the LYM value was found to be higher, and accordingly, the NLR value was significantly lower (Table 2). In addition, RDW, PLT and PDW values were found to be significantly different in HbsAg positive pregnant women (p<0.001). AST and ALT values in the patient group were also higher than the control group (p<0.01). However, no statistically significant difference was observed between the HBsAg positive and negative control groups in terms of vitamin D values (Table 2). No correlation was also found between the vitamin D and ALT in both groups (r=-0.316; p=0.153 in control, r=0.173; p=0.344 in HbsAg positive).

## Influenza Infection

The third group consisted of 63 influenza positive and 67 healthy pregnant women. The mean age of the influenza and control groups was 28.4 ± 6.0 and 28.8 ± 5.5, respectively (p>0.05). WBC, RBC, PLT, NEU, LYM, EOS, BASO, NEU%, LYM%, EOS%, BASO% values of this group were evaluated retrospectively. In addition, LYM x PLT and NLR values were calculated. BASO% values (p<0.001) and NLR values (p<0.01) of the pregnant group with influenza infection were found to be statistically significantly higher than the control group. A more detailed description of the data is given in Table 3. In influenza-infected pregnant women, a weak correlation was also observed for the NLR value and gestational week (r=0.279; p<0.05).

## Covid-19 Infection

In the last group, there were 130 Covid-19 positive and 67 negative pregnant women. The mean age of the influenza and control groups was 26.3 ± 4.2 and 28.8±5.5, respectively

**Table 3.** Comparison of influenza and control groups.

| | Influenza (n=63) | Control (n=67) | p-value |
|---|---|---|---|
| Age | 28.43 ± 6.06 | 28.88 ± 5.54 | [1]0.666 |
| WBC | 9.85 ± 3.06 | 9.96 ± 3.25 | [2]0.608 |
| RBC | 4.19 ± 0.42 | 4.19 ± 0.49 | [1]0.945 |
| PLT | 237.64 ± 55.77 | 237.34 ± 82.2 | [2]0.483 |
| NEU | 7.4 ± 2.5 | 7.21 ± 3.35 | [2]0.422 |
| LYM | 2.1 ± 0.92 | 1.97 ± 0.62 | [2]0.837 |
| EOS | 0.19 ± 0.16 | 0.12 ± 0.09 | [2]0.328 |
| BASO | 0.02 ± 0.02 | 0.04 ± 0.13 | [2]0.858 |
| NEU% | 71.69 ± 9.93 | 71.11 ± 10 | [2]0.610 |
| LYM% | 19.31 ± 8.84 | 21.76 ± 8.59 | [2]0.060 |
| EOS% | 2.13 ± 2.18 | 1.39 ± 1.3 | [2]0.494 |
| BASO% | 0.48 ± 0.32 | 0.23 ± 0.14 | [2]0.000* |
| LYMxPLT | 512.07 ± 314.32 | 481.15 ± 242.09 | [2]0.973 |

White blood cell (WBC), red blood cell (RBC), platelet (PLT), neutrophil (NEU), lymphocyte (LYM), eosinophil (EOS), basophil (BASO), [1]Student t Test, [2]Mann Whitney U Test, all values are shown as mean± SD, SD: Standard deviation, *p<0.05

(p<0.05). AST, ALT, WBC, PLT, NEU, LYM, EOS, BASO, NEU%, LYM%, EOS%, BASO% parameters of the pregnant women were evaluated retrospectively. AST (p<0.001) and ALT (p<0.01) values of pregnant women with Covid-19 infection were found to be statistically significantly higher than the healthy control group (Table 4). In addition, BASO (p<0.001), BASO% (p<0.05) values were found to be statistically significant higher and WBC values (p<0.01) were lower in pregnant women with Covid-19 infection. The detailed information is shown in Table 4. The Covid-19 infection group was also compared with the influenza group (Table 5). Finally, Table 6 summarizes the parameters with significant differences and related groups.

## DISCUSSION

This study aimed to examine the hematological and biochemical parameters of pregnant women who had viral infections during pregnancy such as CMV, HBV, Influenza and Covid-19. The first group consisted of CMV, one of the most common congenital infections. While no difference was found in WBC counts in this group, significant differences were found in ALT, AST and NEU values. CMV-associated infection in fibroblast and macrophage cells causes activation of the nuclear factor kB (NF-kB) (7). NF-κB, is responsible for the activation of various cytokines, and may also be associated with the increase in ALT and AST levels in CMV infection (8).

In the study by Nigro et al., ALT, AST and LYM% were found to be higher in pregnant women with primary and recurrent CMV infection compared to the control group. Accordingly, an increase in AST and at least 40% LYM may indicate the presence of primary CMV infection (9). Interestingly, although a significant increase in ALT and AST values was detected in this study, the increase in LYM% was not significant.

The effects of pregnancy on chronic HBV infection (CHB) or the effects of HBV infection on pregnancy are not clearly known. However, it is possible that changes in the immune system during pregnancy allow for greater viral replication in individuals with chronic HBV. Detection of HBsAg is one of the main serological markers of acute and chronic hepatitis B (10). However, some values in routine blood parameters may also give clues about infection. In this study, AST and ALT values of the HBsAg positive pregnant group were found to be statistically significantly higher than the healthy control group, and these values are consistent with the literature. In addition, several studies have reported high RDW levels in CHB patients (11-13). It has been noted that viral load can have a significant effect on RDW when ALT levels are more than 2 times higher than in the normal group (14). High RDW is positively associated with the severity of CHB disease and may be a determining factor in the follow-up of patients with liver cirrhosis (11-13). Although the increase in ALT levels in this study altered significantly, there was no two-fold difference compared to the control group. Interestingly, the RDW values of the HBsAg positive pregnant group were significantly lower than the control group. Conflicting results regarding the RDW value may be due to different cohorts. On the other hand, anemia and, nutrition, folate and B12 deficiencies in pregnancy may also be the reason for these differences.

**Table 4.** Comparison of Covid-19 positive and control groups.

| | Covid-19 (n=130) | Control (n=67) | p-value |
|---|---|---|---|
| Age | 26.36 ± 4.17 | 28.88 ± 5.54 | [1]0.003* |
| AST | 28.55 ± 33.3 | 16.76 ± 5.21 | [2]0.000* |
| ALT | 25.08 ± 52.69 | 13.97 ± 9.46 | [2]0.002* |
| WBC | 8.64 ± 3.39 | 9.96 ± 3.25 | [2]0.001* |
| PLT | 237.78 ± 85.49 | 237.34 ± 82.2 | [2]0.977 |
| NEU | 10.59 ± 16.59 | 7.21 ± 3.35 | [2]0.067 |
| LYM | 3.1 ± 5.25 | 1.97 ± 0.62 | [2]0.091 |
| EOS | 0.19 ± 0.36 | 0.12 ± 0.09 | [2]0.119 |
| BASO | 0.038 ± 0.15 | 0.037 ± 0.13 | [2]0.000* |
| NEU% | 65.71 ± 18.06 | 71.11 ± 10 | [2]0.087 |
| LYM% | 21.44 ± 10.18 | 21.76 ± 8.59 | [2]0.858 |
| BASO% | 0.31 ± 1.03 | 0.23 ± 0.14 | [2]0.026* |

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), white blood cell (WBC), platelet (PLT), neutrophil (NEU), lymphocyte (LYM), eosinophil (EOS), basophil (BASO), [1]Student t Test, [2]Mann Whitney U Test, all values are shown as mean± SD, and median values are presented in brackets, SD: Standard deviation, *p<0.05.

**Table 5.** Comparison of Covid-19 positive and influenza groups.

| | Covid-19 (n=130) | Influenza (n=63) | p-value |
|---|---|---|---|
| PLT | 237.78 ± 85.49 | 237.64 ± 55.77 | [2]0.459 |
| MPV | 10.15 ± 1.14 | 9.51 ± 1.87 | [1]0.371 |
| PDW | 14.35 ± 2.43 | 15.08 ± 1.77 | [2]0.599 |
| NEU | 10.59 ± 16.59 | 7.4 ± 2.5 | [2]0.330 |
| EOS | 0.19 ± 0.36 | 0.19 ± 0.16 | [2]0.258 |
| NEU% | 65.71 ± 18.06 | 71.69 ± 9.93 | [2]0.041* |
| EOS% | 1.27 ± 1.49 | 2.13 ± 2.18 | [2]0.251 |
| WBC | 8.64 ± 3.39 | 9.85 ± 3.06 | [2]0.002* |
| RBC | 3.98 ± 0.55 | 4.19 ± 0.42 | [1]0.008* |
| LYM | 3.1 ± 5.25 | 2.1 ± 0.92 | [2]0.480 |
| MONO | 0.96 ± 1.74 | 6.55 ± 3.03 | [2]0.000* |
| BASO | 0.04 ± 0.15 | 0.02 ± 0.02 | [2]0.238 |
| LYM% | 21.44 ± 10.18 | 19.31 ± 8.84 | [2]0.111 |
| BASO% | 0.31 ± 1.03 | 0.48 ± 0.32 | [2]0.000* |
| MONO% | 6.16 ± 2.54 | 5.83 ± 2.41 | [2]0.746 |

Platelet (PLT), mean platelet volume (MPV), platelet distribution width (PDW), neutrophil (NEU), eosinophil (EOS), white blood cell (WBC), red blood cell (RBC), lymphocyte (LYM), monocyte (MONO), basophil (BASO), [1]Student t Test. [2]Mann Whitney U Test, all values are shown as mean± SD, and median values are presented in brackets, SD: Standard deviation,*p<0.05

**Table 6.** Representation of the parameters with significant differences in study groups.

| | Group I | | | Group II | Group III |
|---|---|---|---|---|---|
| | **CMV** | **Influenza** | **Covid-19** | **Hepatitis B** | **Covid-19** |
| **AST** | + | NC | + | + | NC |
| **ALT** | + | NC | + | + | NC |
| **WBC** | - | - | + | + | + |
| **RBC** | NC | - | NC | NC | + |
| **NEU** | + | - | - | + | - |
| **NEU%** | NC | - | - | NC | + |
| **BASO** | NC | - | + | NC | - |
| **BASO%** | NC | + | + | NC | + |
| **RDW** | NC | NC | NC | + | NC |
| **PDW** | NC | NC | NC | + | - |
| **NLR** | NC | NC | NC | + | NC |
| **PLT** | NC | - | - | + | - |
| **LYM** | - | - | - | + | - |

Significant ones are indicated with plus, non-significant ones are indicated with minus. NC: not-compared. Group I: CMV vs Control, Influenza vs Control and Covid-19 (+) vs Control; Group II: HBsAg (+) vs HBsAg (-); Group III: Covid-19 (+) vs Influenza. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), white blood cell (WBC), red blood cell (RBC), neutrophil (NEU), basophil (BASO), red blood cell distribution width (RDW), platelet distribution width (PDW), neutrophil lymphocyte ratio (NLR), lymphocyte (LYM), CMV (Cytomegalovirus)

Moreover, the PLT value was found to be significantly higher and the NLR value to be lower in pregnant women with HBV in this study. Recently, the NLR value, which can be easily detected from peripheral blood, has attracted attention as a marker of systemic inflammation. Atay et al. found an increase in NLR rates in the CHB group compared to the controls and in the advanced fibrosis group compared to the mild fibrosis group (15). However, in some recent studies, PLT values were found to be higher in CHBs without cirrhosis and liver failure, and lower NLR values compared to controls (13,16-18). Although it is thought that the increase in the NLR value may be associated with poor prognosis and survival, according to studies conducted with various diseases, the results have not been sufficiently clarified.

The detection of NLR may also play an important role in the fight against Covid-19 and influenza-related infections. Neutrophils show potent antimicrobial effects as important components of the leukocyte population. In addition to triggering the release of multiple cytokines to fight infections, they increase the production of reactive oxygen species (ROS) that can result in tissue damage. Although the antiviral responses of neutrophils are not well known, they are immune effector cells that play a primary role in viral infection sites (19). Routine laboratory findings of pregnant women and other adult patients in the early period of Covid-19 infection are contradictory. However, most of them have normal or decreased WBC, high NLR value and lymphocytopenia (6, 20, 21). The NLR value can be a prognostic biomarker and was reported in various studies including patients with non-severe, mild and severe Covid-19 infections (22, 23). In addition, a meta-analysis showed that individuals with severe Covid-19 have a higher NLR than non-severe ones (24). In various studies conducted in our country, high NLR values have been reported in individuals with severe Covid-19. NLR has attracted attention as a predictive factor for Covid 19 diagnosis, prognosis, and survival (25-27). Yılmaz et al. reported that WBC, NEU and LEU values were significantly lower in Covid 19 positive pregnant women compared to negative ones, but they did not find a significant difference in NLR values (28). However, the NLR value showed significant differences when evaluated according to disease severity (28, 29). On the other hand, a retrospective analysis of a large cohort revealed that the NLR values in the first, second and third trimesters of pregnancy may vary (30). In this context, it is important to know in which trimester the pregnant woman is during the measurement of NLR values. In this study, the WBC count was found to be significantly lower between Covid-19 and control groups, while the increase in NLR and LYM values and the decrease in LYM and NEU percentages were not significant. The reason for this may be asymptomatic or mild symptoms of the disease, or the study group may be composed of pregnant

women in different trimesters. Mild thrombocytopenia, liver enzymes (i.e. ALT and AST), lactate dehydrogenase and C-reactive protein (CRP) are also likely to increase in the early period of Covid-19 infection (31). Less common symptoms, according to the laboratory findings of Guan et al., include elevated ALT, AST, and creatine kinase levels (21). In this study, high AST and ALT values were observed in the pregnant group with Covid-19 infection compared to controls.

On the other hand, the clinical symptoms of Covid-19 and influenza are quite similar. In this context, the data of pregnant patients with Covid-19 and flu were also analyzed to determine the distinguishing parameters in the study. The results obtained from various studies have shown that the NLR and WBC values are remarkably lower in Covid 19 individuals than in the influenza group (5, 26, 32). Consistent with other studies, NLR and WBC values were significantly decreased in pregnant women with infection due to the presence of Covid-19 in this study.

There are 5 subtypes of white blood cells, the number of which increases significantly during infection, consisting of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Unlike the study by Kazancıoğlu et al., a significant increase in BASO and BASO% values were observed in Covid-19 pregnant women in this study (26). Basophils are relatively rare leukocytes that contribute to allergic inflammations due to their ability to migrate from the blood to various other tissues. In addition, there are studies showing that basophils have the capacity to produce Th2-type cytokines and the ability to initiate Th2 immunity (33). In other studies, it has been reported that basophils were decreased during acute disease but were returned to normal after a few days. It has been suggested that basophil counts, which decrease at the beginning of the disease and change afterward, may be an important prognostic marker (34).

## CONCLUSION

Immunomodulation, which mediates tolerance of the allogeneic fetus during pregnancy, may render the pregnant woman more susceptible to viruses. According to this study, WBC values were decreased in Covid-19 positive pregnant women, and BASO% and BASO were increased. However, data on the variation of the basophil values due to viral infections are limited. In addition, the WBC, RBC, NEU%, MONO and BASO values of pregnant women with Covid-19 were lower than those with influenza infection. Finally, in line with the literature, high AST and ALT values in the CMV infected group and increased AST, ALT, PLT and RDW values in HBsAg positive patients may help the prediction of infections. Although these changes in blood parameters give clues about the presence of infection, they are not sufficient to be a distinctive pattern of disease. To understand the hematological and biochemical data relation, the study needs to be improved with various larger patient groups.

## LIMITATIONS

Since this study was evaluated retrospectively, demographic data of pregnant women such as body mass index, weight averages and pregnancy complications could not be obtained. Chronic or acute infection information of pregnant women with HBV infection could not be obtained either.

**Acknowledgments:** We would like to thank Professor Sebahat Aksaray and Professor Nesrin Emekli for their support and critical comments on this study.

**Ethics Committee Approval:** The study protocol was approved from the ethical committee of the Istanbul Medeniyet University Goztepe Training and Research Hospital, Clinical Research Ethics Committee (Decision No: 2021/0140).

**Authors' Contributions:** Conception/Design of Study – M.G.S., D.B., N.E.E.; Data Acquisition – D.B., S.E., M.G.S., N.E.E.; Data Analysis/Interpretation – N.E.E., S.S., M.G.S.; Drafting Manuscript– N.E.E., M.G.S., S.S.; Critical Revision of Manuscript- M.G.S., S.S., D.B., S.E.; Final Approval and Accountability– M.G.S., D.B., S.E., S.S., N.E.E.

**Conflict of Interest:** Authors declared no conflict of interest.

**Financial Disclosure:** Authors declared no financial support.

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Experimed 2023; 13(2): 156-162

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1317269

# The Effect of Otitis Media with Effusion on Language and Cognitive Skills in School Age Children

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**Cite this article as:** Savas M. The effect of otitis media with effusion on language and cognitive skills in school age children. Experimed 2023; 13(2): 156-162.

## ABSTRACT

**Objective:** Otitis media with effusion (OME) is a common condition in childhood and can interfere with cognitive development. The fact that it can be easily overlooked causes it to become chronic and has negative consequences in the long term (1). The negative consequences of OME in terms of speech and language disorders include speech sound disorders and developmental language disorders. For this purpose, cases admitted to the speech and language therapy clinic were screened for OME consecutively, and its relationship with language disorders was analyzed.

**Materials and Methods:** 50 children aged 8 to 10 years without mental retardation and hearing impairment were evaluated with audiology, language, repetition (non-word and sentence), and visual perceptual tests. In addition to standard language assessment, narrative samples were obtained and analyzed which is considered a descriptive approach.

**Results:** 26 children were positive for OME. Children with OME scored lower on language and repetition tests. There was no significant difference between the groups with and without OME in terms of syntactic complexity, narrative skills and visual perceptual performance. The Mann-Whitney U test was used for the comparison of parameters between groups ($p<0.05$).

**Conclusion:** The presence of OME negatively affected language development. However, the structural complexity dimension of storytelling, complex sentence production performance, and visual perceptual skills were not affected negatively by OME. Although OME positivity is not accompanied by mental retardation and developmental delay, language development and verbal working memory may be negatively affected. OME should be routinely screened in childhood and should be addressed more closely in children with speech and language disorders.

**Keywords:** Otitis media with effusion, language development, speech sound disorder

## INTRODUCTION

Otitis media with effusion (OME) or serous is an inflammation of the middle ear mucosa characterized by fluid accumulation in a preserved tympanic membrane and cavum tympani without signs and symptoms of acute infection (1). OME is extremely common in early childhood due to an immature immune system, frequent upper respiratory tract infections, and a shorter and more horizontal Eustachian tube than in adults (2). When OME becomes chronic, it can lead to hearing loss, cognitive dysfunction, behavioral problems, and academic failure, resulting in a reduced quality of life (3). When children with a history of chronic OME were compared with their healthy peers in terms of language development and auditory processing performance, articulation and phonemic discrimination skills were found to be negatively affected (4). Similarly, it was found that the longer the duration of the presence of OME in the cavum tympani, the more negatively expressive language skills were affected (5). The impairment of the acoustic-phonetic properties of auditory signals due to OME can lead to an incomplete and inaccurate encoding of speech sounds into phonological working memory and to the misconstruction of mental

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**Submitted:** 20.06.2023 **Revision Requested:** 26.07.2023 **Last Revision Received:** 28.07.2023 **Accepted:** 08.08.2023 **Published Online:** 09.08.2023

representations of words. Children with OME may not hear or could mis-hear short-duration and low-intensity morphemes (6). Since OME may lead to language-cognitive developmental delays, its presence in children with speech and language disorders should be investigated and its relationship with affected language areas should be demonstrated (7). For this purpose, school-age children admitted to a speech and language therapy clinic were screened consecutively for OME, and the effect of middle ear inflammation with effusion on the components of language was analyzed.

Language, as a basic expression of the human mind, is a code system that enables the emergence and transmission of thoughts and the continuity of communication between people (8). Semantic knowledge of concepts, actual use of words, and connotative/symbolic transfer of words constitute the semantic component of language (9). The syntactic/morphosyntactic side is the arrangement of words to form meaningful and regular sentences (10). Pragmatics refers to the full range of skills required for communicative purposes in the social context of language (11). Linguistic processing is closely related to executive functions such as planning, reasoning, abstraction, working memory, and inhibition (12). Turkish is a suffixal and morphologically rich language (13). Morphemes have critical importance in language comprehension and speech, and morphological processing is closely related to cognitive processes (14). The multilayered nature of language makes linguistic assessment difficult in clinical settings. Therefore, in addition to standardized tests, repetition tests (sentence and nonword repetition tests) and narrative tools that address the pragmatic dimension should be used.

The aim of this study was to draw attention to the importance of audiological evaluation and linguistic cognitive examination in school-age children to identify possible risks such as cognitive dysfunction, academic failure and social communication difficulties due to OME.

## MATERIALS AND METHODS

### Participants

50 monolingual Turkish-speaking children aged 8 to 10 years (23 girls, 27 boys; mean age 8.78±0.73 years) were included in the study. Ethics committee approval and voluntary consent were obtained from Istanbul Atlas University Ethic Committee (14.03.2023-24890) and in compiance with the Declaration of Helsinki. Inclusion criterias were an absence of developmental delay and primary neurological psychiatric diagnosis, a score of 100 or above on the Wechsler Intelligence Scale for Children-Revised (WISC-R), absence of symptoms of acute otitis media, and right and left ear pure tone hearing averages within the normal range (0-15 dB).

Acute otitis media includes otorrhea of middle ear origin, otalgia, middle ear fluid or effusion, and symptoms of acute local or systemic disease (15). Acute otitis media may also present with a type B tympanogram and is not defined as OME if

**Table 1.** Demographical and Clinical Information of Participants

| | EOM | Age | Frequency | Percentage |
|---|---|---|---|---|
| Age | OME- | 8 | 10 | 41.7 |
| | | 9 | 9 | 37.5 |
| | | 10 | 5 | 20.8 |
| | | Total | 24 | 100 |
| | OME+ | 8 | 10 | 38.5 |
| | | 9 | 12 | 46.2 |
| | | 10 | 4 | 15.4 |
| | | Total | 26 | 100 |
| | OME | SSD | Frequency | Percentage |
| SSD | OME- | SSD+ | 13 | 54.2 |
| | | SSD- | 11 | 45.8 |
| | | Total | 24 | 100 |
| | OME+ | SSD+ | 17 | 65.4 |
| | | SSD- | 9 | 34.6 |
| | | Total | 26 | 100 |
| | OME | LD | Frequency | Percentage |
| LD | OME- | LD+ | 1 | 4.2 |
| | | LD- | 23 | 95.8 |
| | | Total | 24 | 100 |
| | OME+ | LD+ | 10 | 38.5 |
| | | LD- | 16 | 61.5 |
| | | Total | 26 | 100 |
| | OME | Gender | Frequency | Percentage |
| Gender | OME- | Female | 11 | 45.8 |
| | | Male | 13 | 54.2 |
| | | Total | 24 | 100 |
| | OME+ | Female | 12 | 46.2 |
| | | Male | 14 | 53.8 |
| | | Total | 26 | 100 |

OME: otitis media with effusion; OME+: Presence of otitis media with effusion; OME-: Absence of otitis media with effusion; SSD: Speech sound disorder; SSD+: Presence of speech sound disorder; SSD-: absence of speech sound disorder; LD+: Presence of language disorder; LD-: Absence of language disorder.

clinical signs of infection are present (16). Type B tympanogram with a flat curve and normal canal volume is considered diagnostic criteria of OME (17). The otolaryngologist diagnosed OME by considering the clinical examination and bilateral type B values together. Children with bilateral type B results were grouped as otitis media with effusion positive (OME+); children with bilateral type A were grouped as otitis media with effusion negative (OME-).

Participants whose equivalent test score according to the Turkish Articulation and Phonology Test (SST) was below their chronological age were diagnosed as speech sound disorder (SSD). The verbal language composite score (VLSC), obtained from the Test of Language Development-Primary-Fourth Edition Turkish Version (TOLDP-4:T) was used to determine language development. Children with very poor, weak, and below-average VLSC scores were classified as language disorder (LD+), and children with average, above-average, advanced, and very advanced VLSC scores as an absence of language disorder (LD-).

Table 1 shows the distribution of clinical and demographic data.

## Instruments

## Audiological Assessment

Tympanometry is a technique to determine whether the ventilation and ossicles in the middle ear are normal. Abnormal tympanometry values indicate that the patient has a problem with the middle ear. In tympanometry, the response of the eardrum to different pressures and the pressure in the middle ear are evaluated. The results of tympanometry are categorized into three categories: harmonious movement of the eardrum against different pressure values (Type A); presence of fluid in the middle ear/perforation of the membrane (Type B); and cases of negative pressure in the middle ear due to a problem in the Eustachian tube/end stage of ear infection/allergy-related obstruction (Type C) (18).

Pure tone audiometry is used to determine the degree of hearing loss by recording the thresholds at which acoustic stimuli of different intensities are heard at frequencies of 125, 250, 500, 750, 1000, 1500, 2000, 3000, 4000, 6000 and 8000 hertz. According to pure tone average results, hearing loss grades are classified as normal hearing (-10 to 15 dB), very mild (16 to 25 dB), mild (26 to 40 dB), moderate (41 to 55 dB), moderate to severe (56 to 70 dB), severe (71 to 90 dB), very severe (> 90 dB) (19).

## Language Assessment

TOLDP-4:T is a norm-referenced and standardized test that measures receptive and expressive language skills in children aged 4 to 8 years. It consists of 9 subtests: picture vocabulary, relational vocabulary, oral vocabulary, syntactic understanding, sentence repetition, morphological completion, word discrimination, word analysis and word articulation. By converting the raw scores of certain subtests into standard scores, the VLSC which consists of the composite score of listening, speaking, organizing, semantics, grammar, and the sum of all of them, is obtained. According to the VLSC, language development is classified as very poor (<70), poor (70-79), below average (80-89), average (90-110), above average (111-120), advanced (121-130) and very advanced (>130) (20). The reliability and validity scores of the TOLD-P:4: T has been conducted up to the age of 8 years and 11 months, and there is not yet a standardized test to assess the language development of Turkish-speaking individuals over the age of 8 years and 11 months in Turkey. 8 years and 11 months normative values were used for elder children. Since there were participants over the age of 8 years and 11 months in the study, both the VLSC score and whether there was a language disorder according to the VLSC score were categorized and a comparison was made between the groups.

Turkish Nonword Repetition Test *(*TNRT*)* performance is indexed to phonological working memory capacity and auditory attention skills. The TNRT is a practical tool used to distinguish children with developmental language disorder/risk from normally developing children. The test items are played to the participant once and then asked to repeat them. The correctly produced item is scored as 1 and the total number of correctly produced items is used as the score obtained from the test (21). In Turkey, there is no standardized test developed to determine phonological working memory capacity. Validity and reliability studies of the TNRT in Turkish-speaking school-age children are ongoing. However, there are studies comparing various disease groups and healthy subjects using TNRT in Turkish-speaking school-age children (22-23).

The Turkish Multilingual Sentence Repetition Test (LITMUS-TR) includes sentences in 5 different categories: subject-object-predicate sequences, what-whom questions, noun phrases, relative clauses, clausal adjuncts, conditional structures, and assesses morphosyntactic skills. Consisting of 30 sentences, LITMUS-TR is administered by repeating the sentence heard once. When the sentence is repeated completely correctly, it is scored as 1; otherwise, it is scored as 1 (24-25). Several studies have shown that sentence repetition tests can distinguish between children with language impairment and typical language development (26). Sentence repetition tests are therefore part of a group of language tests commonly used by clinicians to assess children's language skills. The TOLD-P:4: T includes a sentence repetition subtest, but compared to the LITMUS-TR, the sentences are longer, more complex, and contain more items. The sentences in LITMUS-TR are structured to contain a minimum of 11 and a maximum of 14 syllables and a minimum of 4 and a maximum of 6 words. In this way, the load on verbal working memory was balanced and the determinants of morphosyntactic structures on correct repetition performance were emphasized. LITMUS-TR normative studies in Turkish-speaking school-age children are ongoing.

## Assessment of Speech Sound Disorder

The SST is a test that is used for screening and differential diagnosis of children with articulation and phonological problems and for which validity, reliability and standardization studies have been conducted. The Articulation Subtest measures the production of 24 phonemes in various positions in the word and the seven most common consonant phrases in Turkish in the context of picture naming. The auditory discrimination test measures the auditory-visual discrimination of 21 consonant phonemes in the smallest monosyllabic word pairs according to their phoneme position, form, and voicing/nonvoicing features. For example, the sounds /k/ and /t/ are different in terms of phoneme position and form, but they share the same in articulatory features (27).

## Assessment of Narrative Skills

The Turkish version of Multilingual Assessment Instrument for Narratives-TR (MAIN) is one of the effective tools for analyzing the micro- and macrostructural features of narrative in children. By analyzing the narrative sample, it makes it possible to evaluate the semantic, morphosyntactic and pragmatic components of language. In this study, the number of complex sentences (main clause with a subordinate clause formed with a verb or inflected verb) productions interpreted in favor of syntactic maturation (28). The number of completed episodes (goal, attempt, outcome [GAO]), is considered the most effective indicator of story complexity (29).

## Assessment of Visual Perceptual Motor Skills

The Bender-Gestalt Visual Motor Perception Test (BG), developed by Bender in 1938, measures the perception of visual stimuli and the ability to integrate visually perceived information into motor systems. In this test consisting of 9 drawings, each figure is placed on a separate page and it is asked to draw the pictures shown one by one in order. There is no time limit for the drawings, which are drawn sequentially using a single A4 size paper. A pencil and eraser are used for drawing. Preliminary Turkish validity and reliability studies were conducted by Ozer (30). The total score was obtained by calculating the participants' error scores. The child's drawings are scored according to the number of errors (30).

## Statistical Analysis

The distribution of variables was calculated by Shapiro Wilk test. Likewise, two group comparisons were performed with Student t or Mann-Whitney U tests. All data are presented as mean ± standard deviation (SD), and p-values. Statistical analyzes were performed with SPSS IBM 25.0 and p-values <0.05 were considered significant.

## RESULTS

Table 2 shows the cognitive and linguistic parameters of the participants. There was no significant difference between OME+ and OME- groups according to age (p>0.05). TNRT value of OME- group was significantly higher than OME+ group (p<0.05). The LITMUS-TR value of the OME- group was significantly higher than the OME+ group (p<0.05). There was no significant difference between OME+ and OME- groups according to BG, SSD, complex sentence, and GAO (p>0.05).

**Table 2.** Comparison of cognitive and linguistic parameters of the participants

| | OME | N | Mean | SD | Z | p |
|---|---|---|---|---|---|---|
| Age | OME+ | 26 | 8.77 | 0.71 | -0.042 | 0.967 |
| | OME- | 24 | 8.79 | 0.779 | | |
| VLSC | OME+ | 26 | 92.46 | 13.189 | -4.002 | **0.001*** |
| | OME- | 24 | 108.83 | 10.218 | | |
| SSD | OME+ | 26 | 0.65 | 0.48 | -0.08 | **0.423** |
| | OME- | 24 | 0.54 | 0.5 | | |
| LD | OME+ | 26 | 0.38 | 0.49 | -2.895 | **0.004*** |
| | OME- | 24 | 0.41 | 0.2 | | |
| TNRT | OME+ | 26 | 8.5 | 2.486 | -3.911 | **0.005*** |
| | OME- | 24 | 11.79 | 2.449 | | |
| LITMUS-TR | OME+ | 26 | 16.46 | 4.658 | -4.523 | **0.002*** |
| | OME- | 24 | 23.75 | 4.089 | | |
| BG | OME+ | 26 | 9.27 | 4.618 | -1.141 | 0.254 |
| | OME- | 24 | 10.58 | 4.211 | | |
| Complex Sentence | OME+ | 26 | 4.08 | 1.129 | -0.425 | 0.671 |
| | OME- | 24 | 3.92 | 0.929 | | |
| GAO | OME+ | 26 | 0.5 | 0.51 | -1.147 | 0.251 |
| | OME- | 24 | 0.71 | 0.624 | | |

OME: otitis media with effusion; OME+: Presence of otitis media with effusion; OME-: Absence of otitis media with effusion; SSD: Speech sound disorder; VLSC: The verbal language composite score; TNRT: Turkish Nonword Repetition Test; LITMUS-TR: The Turkish Multilingual Sentence Repetition Test; BG: The Bender-Gestalt Visual Motor Perception Test; GAO: Goal, attempt, outcome.

The VLSC value of the OME- group was significantly higher than the OME+ group (p<0.05). The LD frequency of the OME+ group was significantly higher than the OME- group (p<0.05).

## DISCUSSION

Various studies have shown that unilateral hearing loss negatively affects language and cognitive functions in children. (31). Similarly, the presence of middle ear cavity effusion without hearing loss may impair the perception of

phonetic and phonological features of speech sounds (32). In our study, the presence of isolated OME without hearing loss negatively affected language skills. There is a lot of evidence that OME is comorbidity with the SSD (33). Similarly, SSD was seen with a higher frequency in OME+ cases included in this study. Since SSD is the clinical counterpart of articulation disorder and auditory discrimination problems, it can cause social communication difficulties by negatively affecting the intelligibility of the individual (34). This situation can be considered as a finding that supports the need for OME screening in cases with SSD.

In this study, school-age children with and without OME were compared in terms of language skills and visuospatial functions. BG is sensitive to measuring the level of maturation necessary for the integration of the visual perceptual process into motor skills (35). BG performance is not affected by the presence of OME. The incidence of language disorders was found to be higher in the OME+ group. However, since children older than TOLD-P:4:T normative data were included, a comparison was made according to VLSC scores. VLSC scores were found to be lower in the OME+ group. Therefore OME+ can be considered a risk factor for language disorder. Descriptive analyzes are needed due to the fact that standard language assessments are norm dependent, do not provide clues for the use of language in social contexts, and are insufficient to reveal the strong and weak language skills of children who are unfamiliar with the test or who come from different cultural/ethnic backgrounds (36). For this reason, we used MAIN-TR to assess the children's storytelling and narrative skills in terms of complex syntactic production and story complexity.

Syntax, the structural basis of language, is defined as the organization/sequencing of words in sentences to express thoughts, feelings, preferences, and observations in a clear, precise, and efficient way. Syntax emerges in early childhood and continues to develop throughout childhood, adolescence, and early adulthood. With development, sentence lengths increase and sentences contain more dependent clauses (37). This increase in the number of clauses embedded in the main clause (complex sentence production) and their addition to independent clauses is considered a consequence of language maturation (38). The data obtained in this study show that the presence of OME does not lead to morphosyntactic involvement in expressive language.

MAIN-TR consists of stories with pictures in which the actions of the protagonists are clearly described. The narrator has to infer the character's motivation and triggering situations by interpreting the actions in the pictures. The inclusion of statements about the purpose of the protagonist's actions (Goal), the realization of the intended action (Attempt), and the outcome of the action (Outcome) within a story section (GAO sequences) is considered a structural complexity parameter. GAO is the highest level indicator of coherent storytelling ability (39). OME has no direct effect on the ability to produce plots based on temporal and causal relationships.

## Clinical Significance of Repetition Tests

Sentence comprehension tasks in standard language assessment tools require the visual-spatial perception of pictures, storing the entire sentence in working memory to reach its final meaning, and directing attentional resources to the target in order to select the picture most relevant to the meaning. In sentence repetition tests, it is accepted that the cognitive load is relatively less because there is no visual processing and semantic judgment for the repetition of the heard sentence. Therefore, sentence repetition tests are preferred in children with language impairment in order to control the reflection of cognitive dysfunctions on language as much as possible. Repetition of short sentences may not require the use of working memory and implicit grammatical acquisition. However, long sentences may exceed memory capacity. The inability to repeat the sentence exactly may not be due to morphosyntactic difficulties but to the length of the target structure (40). For this reason, the sentences in LITMUS-TR were controlled in terms of sentence length, number of phrases, number of words/ suffixes and number of syllables in each sentence (41). In our study, it was observed that the LITMUS-TR sentence repetition scores of OME+ subjects were lower. It seems that OME has a negative effect on the morphosyntactic processing needed in sentence repetition.

Nonword repetition tests can measure the capacity of phonological processing and phonological working memory, which are considered critical skills in new word acquisition. In order to repeat a meaningless word without error, acoustic representations must be formed. Similarly, when a new word is heard for the first time, acoustic signals must be stored in auditory working memory until a connection is made between the real-world referent of the word and its phonological representation (42). In the case of OME, nonword performance is becoming poor. It leads to the conclusion that OME causes difficulties in the construction and storage of phonotactic and phonological representations of auditory verbal signals. In various studies, it has been revealed that EOM negatively affects verbal working memory performance (43-44). There are no studies directly addressing the relationship between EOM and repetition tests. The low scores of the EOM+ group in the LITMUS-TR and TNRT tests may be related to the determinant role of working memory on repetition performance.

In conclusion, OME causes language impairment despite normal hearing thresholds and negatively affects morphosyntactic and phonological processing. It has no effect on storytelling skills and visual-spatial copying performance. Although OME is one of the most common clinical pictures encountered by physicians providing outpatient clinic services for children, (32) easily overlooked and negatively affects the course of development in childhood (16). Therefore, OME should be routinely screened and treatment indications should be evaluated in childhood.

## Limitations

One of the key limitations was the insufficient number of participants, which might have affected the statistical power and generalizability of the findings. Additionally, due to the inability to access the previous infectious otitis media history of the OME+ group, the formation of chronic and acute OME+ subgroups was not feasible. Furthermore, although the study included participants with type B tympanometry, a recognized indicator of OME, the lack of information regarding the frequency of previous infectious otitis media in the sample remains a constraint that needs to be addressed in future research. These limitations emphasize the importance of a cautious interpretation of the results and underscore the need for larger sample sizes and comprehensive data collection in future investigations on this subject.

**Ethical Approval:** Ethical approval of the study was obtained from Istanbul Atlas University Local Ethics Committee (14.03.2023-24890).

**Ethical Committee Approval:** N/A.

**Conflicts of Interest:** The author declare no conflict of interest.

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

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# EXPERIMED

## AIMS AND SCOPE

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EXPERIMED

## INSTRUCTIONS TO AUTHORS

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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.

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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).

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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

**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 |

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.

**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 Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık 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.

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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.

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