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Peripheral Expression Levels of Selected Oxidative Stress-Related Genes in Alzheimer's Disease

Alzheimer Hastalığında Oksidatif Stres ile İlişkili Seçilmiş Genlerin Periferik Kandaki Anlatım Düzeyi

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ABSTRACT

Objective: The etiology of Alzheimer's disease (AD) is affected via oxidative stress. Antioxidant enzymes are extremely important in preventing reactive oxygen species (ROS) causing damage in the cell. The changes in expression levels of oxidant and antioxidant genes are key factors in cell response to oxidative stress. As a result, this study investigated the change in expression levels of specific oxidative stress related genes (*solute carrier family 7 member 11 (SLC7A11), glutathione peroxidase 4 (GPX4), catalase (CAT)* and *acyl-coa synthetase long chain family member 4 (ACSL4)*) in peripheral blood of AD patients.

Material and Method: Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to assess the expression levels of oxidative stress-related genes in 25 AD patients and 22 controls, and the findings were statistically evaluated.

Results: *SLC7A11, GPX4, CAT,* and *ACSL4* gene expression levels did not vary significantly between AD patients and controls. The results also showed significant negative correlation between age of onset and *ACSL4* expression.

Conclusion: This is the first study that evaluated mRNA expression levels of *SLC7A11, GPX4* and *ACSL4* genes in AD. The results suggested that the peripheral blood expression of above-mentioned genes did not alter in AD. However, due to the small number of subjects, this findings are preliminary and should be validated with a larger number of subjects.

Keywords: Alzheimer's Disease, oxidative stress, *SLC7A11, GPX4, CAT, ACSL4*

ÖZ

Amaç: Oksidatif stres, Alzheimer hastalığının (AH) etiyolojisinde önemli bir rol oynamaktadır. Antioksidan enzimler reaktif oksijen türevlerinin (ROS) hücreye zarar vermesini önlemek için çok önemlidir. Oksidan ve antioksidan enzimleri kodlayan genlerin anlatım seviyelerindeki değişimler hücrenin oksidatif strese karşı verdiği yanıtta anahtar faktördür. Bu nedenle çalışmamızda, AH hastalarının periferik kanlarında spesifik oksidatif stres ile ilişkili genlerin (*solut taşıyıcı aile 7 üye 11 (SLC7A11), glutatyon peroksidaz 4 (GPX4), katalaz (CAT) ve açil-koA sentetaz uzun zincir aile üyesi 4 (ACSL4)*) anlatım düzeylerindeki değişimin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Periferik kan lökositlerinde oksidatif stresle ilgili genlerin ekspresyon düzeylerindeki değişiklikler 25 AH hastası ve 22 kontrolde kantitatif ters transkripsiyon polimeraz zincir reaksiyonu (qRT-PCR) ile belirlenmiş ve sonuçlar istatistiksel olarak değerlendirilmiştir.

Bulgular: *SLC7A11, GPX4, CAT* ve *ACSL4* genlerinin anlatım düzeyleri, AH hastaları ve kontroller arasında istatistiksel olarak bir farklılık göstermemiştir. Ayrıca, sonuçlarımız başlangıç yaşı ile *ACSL4* ekspresyonu arasında anlamlı negatif bir korelasyon olduğunu göstermiştir.

Sonuç: Araştırmamız, AH hastalarının periferik kanlarında *SLC7A11, GPX4* ve *ACSL4* genlerinin anlatım düzeylerini değerlendiren ilk çalışma olup, sonuçlarımız söz konusu genlerin periferik kandaki anlatımlarının AH'de değişmediğini göstermiştir. Bununla birlikte, az sayıda hasta ve kontrol nedeniyle, bu bulgular başlangıç niteliğindedir ve daha geniş çalışma gruplarında doğrulanması gerekmektedir.

Anahtar Kelimeler: Alzheimer hastalığı, oksidatif stres, *SLC7A11, GPX4, CAT, ACSL4*

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease marked by increasing cognitive dysfunction, as well as accumulated amyloid plaques and neurofibrillary tangles in the brain. (1). The increase in the prevalence of AD is one of the major health problems. Loss of synaptic communication and neuronal death are among the main underlying causes of AD (2). However, the mechanisms that underpin neuronal death in AD are not fully understood and that holds back the improvement of efficient therapeutic approaches. Data from several studies suggests that approximately 80% of AD cases are due to genetic factors, based on twin and family studies (3,4). In addition to genetic factors, environmental factors are also very important in a sporadic form of AD, especially after the age of 65 (5). The oxidative stress is a common condition that has considerable impact on AD etiology. Oxidative stress causes oxidative modifications in nuclear and mitochondrial DNA in AD. Increased reactive oxygen species (ROS) formation, mitochondrial dysfunction, an impaired antioxidant activity, or a combination of these elements are the most common causes (6). ROS can act as a signal molecule or alter gene expression of signal molecules within the cell. Increased ROS residuals may affect the molecular mechanism of synaptic activity and neurotransmission causing cognitive dysfunction (7). Also, oxidative stress is associated with the deposition of β-amyloid (Aβ) plaque (8), while Aβ plaque leads to the formation of free radicals and oxidative stress (9). Against oxidative stress, the body uses its own defense mechanism through antioxidants. Changes in the expressions of antioxidant enzyme genes are important for the cell response to oxidative damage (10). One of these antioxidant enzymes is glutathione peroxidase 4 (GPX4) which needs glutamate, cystine and cysteine for enzyme activation (11). Catalase (CAT), encoded by the *CAT* gene, is another important antioxidant enzyme that basically converts two molecules of hydrogen peroxide into one molecule of oxygen and two molecules of water in a two-step reaction (12). A study performed by Habib et al. showed catalase-amyloid interactions in neurotoxic Aβ peptides stimulated oxidative stress (13). Solute carrier family 7 member 11 (SLC7A11) has an important function in the antioxidant mechanism as it supports the survival and growth of the cell by providing cystine uptake and promoting glutathione synthesis under oxidative stress (14). Ferroptosis, a recently identified mechanism of cell death, has also been shown to play a role in oxidative stress and neurodegenerative disorders in recent years. The catalytic enzyme acyl-coa synthetase long chain family member 4 (ACSL4) is involved in the ferroptosis lipid metabolism pathway. The ACSL4 establishes cell susceptibility to ferroptosis and leads to ferroptotic cell death (15).

The function of antioxidant enzymes is mostly regulated at the transcriptional level. Inability to produce sufficient antioxidant enzyme mRNA against ROS accumulation causes the cell to be unable to defend itself against oxidative stress. Variations in antioxidant gene expression levels can lead to oxidative harm in AD patients' central nervous systems (10). If oxidative stress increases in AD, an increase in the function of antioxidant enzymes and gene expressions can be expected in AD patients. Yet, conflicting results have been obtained in postmortem studies in AD brains on antioxidant gene expressions (16) and there are few studies showing the alteration of antioxidant gene expression levels in AD (10). This study investigated whether *GPX4, CAT, SLC7A11* and *ACSL4* gene expressions, which are known to be associated with oxidative stress, changed in the peripheral blood of AD patients.

MATERIAL AND METHOD

Study Population

The research involved 25 AD patients and 22 healthy controls who had no background of significant neurologic or mental illness. Participants were recruited from Istanbul Faculty of Medicine, Istanbul University in the Behavioral Neurology and Movement Disorders Unit of Neurology Department. The diagnosis of dementia was made according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders (NINCDS-ADRDA) criteria. All participants signed informed consent forms. The Istanbul University's Ethics Committee gave approval to the study's procedures.

Genetic Analysis

Total RNA was collected from leukocytes in the peripheral blood using trizol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The iScript™ cDNA synthesis kit (Bio-Rad, CA, USA) was used to synthesize cDNA from 1µg of total RNA according to the manufacturer's protocol. The SYBR Green I Assay (Thermo Fisher Scientific, MA, USA) was used in a qRT-PCR on the Lightcycler 480 Real-Time PCR system (Roche, Basel, Switzerland). The data was normalized using *GAPDH* as the housekeeping gene, and the relative expression levels of *SLC7A11, GPX4, CAT,* and *ACSL4* genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Data Analysis

Expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* among groups were compared by the non-parametric two-tailed Mann–Whitney U test. Spearman's rho test was performed to investigate correlations between *SLC7A11, GPX4, CAT* and *ACSL4* mRNA expressions and age, age at onset, and mini mental state examination (MMSE) scores. The categorical variables were compared using Fisher's exact test. A p value was considered statistically significant for less than 0.05. All statistical analysis was performed using SPSS Statistics 23.0 software (IBM Corp., USA).

RESULTS

Table 1 summarizes the descriptive characteristics of the study population. There was a significant age gap between AD patients and controls, and the overall MMSE score in patients was significantly lower than in controls (Table 1).

The expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* genes

Table 1. Descriptive characteristics of study population.

	AD (n=25) (mean±SD)	Control (n=22) (mean±SD)	p-value
Age, years, (range)	77.20±5.058, (67-89)	73.90±6.265, (66-91)	**0.035**
Age of onset, years, (range)	72.20±5.848, (65-84)	-	
Gender, % (n)			0.452
Male	48% (13)	59.1% (13)	
Female	52% (12)	40.9% (9)	
MMSE score (range)	18.39±5.255, (10-26)	27.72±3.232, (17-30)	**< 0.0001**

Mann Whitney-U test was used to compare means and Fisher's exact test for percentages, p values in bold shows statistical significance.
AD: Alzheimer's disease, MMSE: Mini Mental State Examination, SD: standart deviation, n: number of individuals

in leukocyte did not vary significantly between AD patients and control subjects. As shown in Figure 1a, the *SLC7A11* expression level in AD (mean±SEM; 1.21±0.18) was similar to controls (1.12±0.16, p=0.819). Although *GPX4* expression seems to be increased in AD (1.89±0.17) as compared to controls (1.56±0.20), no significant difference was found (p=0.207, Figure 1b). In addition, lower *CAT* expression was observed in AD patients (0.89±0.10) as compared to controls (1.09±0.09) without any significance (p=0.72, Figure 1c). Finally, the *ACSL4* expression appears to be higher in AD patients (2.42± 0.25) as compared to controls (2.19±0.16, Figure 1d) without statistical significance (p=0.889).

In AD patients, a correlation analysis was used to show the relationship between age, age of onset, MMSE score, and the expression levels of *SLC7A11, GPX4, ACSL4* and *CAT* genes. The *ACSL4* expression and age at onset had a significant negative correlation, according to our findings (r=-0.458, p=0.028; Figure 2). Other gene expressions were found to have no significant correlation with age, age of onset, or MMSE score in AD patients (Supplementary Figure 1 and 2).

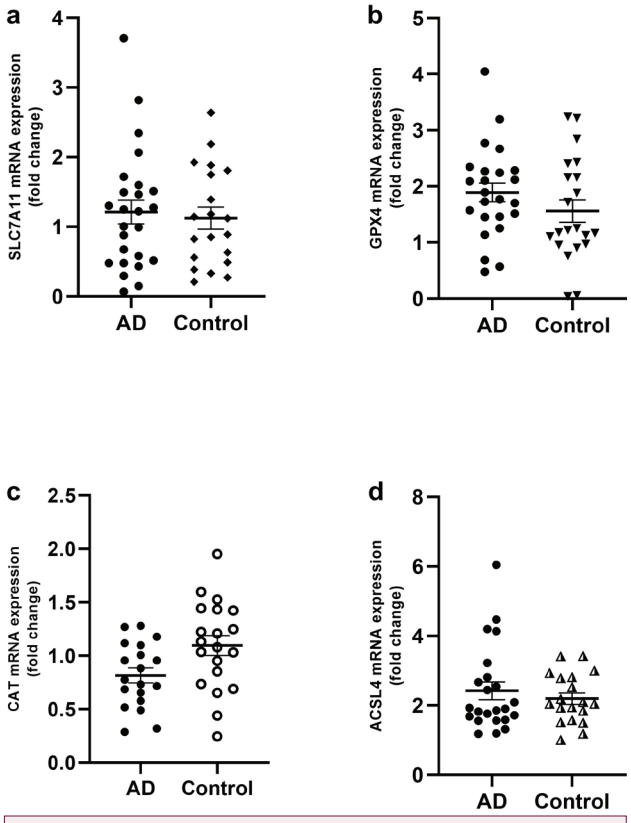

Figure 1. mRNA expression levels in study population **(a)** *SLC7A11* expression in AD and control subjects **(b)** *GPX4* expression in AD and control subjects **(c)** *CAT* expression in AD and control subjects **(d)** *ACSL4* expression in AD and control subjects. Data are presented with mean±SEM, AD Alzheimer disease, SEM standard error of the mean.

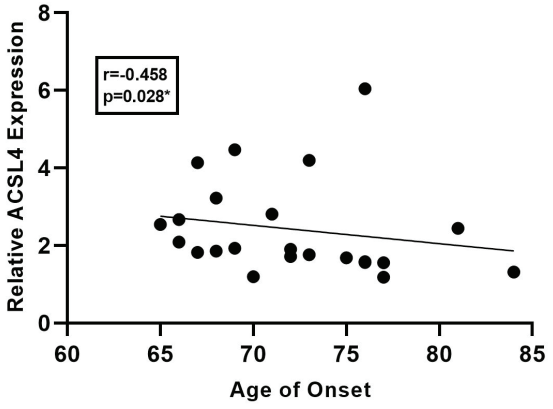

Figure 2. Correlation between *ACSL4* mRNA expression and age of onset in AD patients.

DISCUSSION

Several reports have shown the relationship between oxidative stress and AD etiology (7-9). Oxidative stress tends to increase in the brain with age. Even so, the role of oxidative stress in the etiology of AD is still unknown. The main goal of this research was to investigate whether the expression levels of specific oxidative stress-related genes could be identified in peripheral blood of AD patients. For this purpose, a study was made of the peripheral expression levels of *SLC7A11, GPX4, CAT* and *ACSL4* genes that were known to play a role in oxidative stress.

Cell death by apoptosis is known to occur in response to oxidative stress, and GPX4 has been identified as a key regulator of apoptosis in response to oxidative stress (17). In transgenic mice, increased expression of *GPX4* preserves neurons from oxidative stress and amyloid toxicity (17). Furthermore, in a study by Hambright et al., inactivation of *GPX4* gene in neuronal cells promotes hippocampal neurodegeneration and cognitive dysfunction in a mouse model (18). Studies investigating the relationship between *GPX4* and AD were limited to animal models, so far there has been only one study in humans, and that study explored the effect of *GPX4* polymorphism on episodic memory (19). Therefore, this is the first study to investigate *GPX4* gene expression levels in AD patients. The results showed that *GPX4* expression increased in the peripheral blood of AD patients, although it was not statistically significant. This increase may be the result of neuroprotective effect of *GPX4* in AD.

SLC7A11 plays a crucial role in antioxidant protection by mediating cystine uptake, promoting glutathione synthesis, and sustaining cell viability against oxidative stress conditions (14). In the central nervous system (CNS), glutamate export is particularly important since it is a non-vesicular pathway of release for this excitatory neurotransmitter, which can play a role in neuronal signaling or excitotoxic pathology (20). One study showed that by releasing glutamate through microglia increased Aβ toxicity which suggested that since the *SLC7A11* gene was expressed not only in cultured microglia but also in the reactive microglia inside or around amyloid plaques in transgenic mice, this could be important to Aβ toxicity in AD (21). In a study conducted in the peripheral blood of schizophrenia patients, the significant reduction of *SLC7A11* gene expression supports the hypo-glutamatergic neurotransmission hypothesis in the pathogenesis of schizophrenia and the role of SLC7A11 in neurological diseases (22). However, *SLC7A11* gene expression levels in the peripheral blood of AD patients has not been investigated so far. Although the results showed that the peripheral blood expression of *SLC7A11* did not change in AD, this should be considered preliminary because of the small size and should be further investigated in a larger study.

Catalase is an antioxidant enzyme which is involved in oxidative stress mechanism (12). It has been suggested that catalase deficiency or degradation is implicated in the pathogenesis of degenerative diseases including diabetes mellitus, cancer, AD, Parkinson's disease and hypertension which increase in frequency with age (23). In the analysis of different brain regions of AD patients, Aksenov et al. found a substantial increase in *CAT* expression in brain regions affected by AD pathology (9). This suggested that this antioxidant enzyme could play an important role in protecting CNS cells from oxidative stress in AD. Also, the same study showed that an increased expression of *CAT* leads to an increased tolerance of cultured neural cells to Aβ toxicity (10). In another study conducted in late-onset AD (LOAD) patients, it was observed that *CAT* gene expression levels in peripheral blood were significantly decreased compared to the controls, but the erythrocyte CAT enzymatic activity was significantly increased in LOAD patients. This suggests that even as the expression of antioxidant genes decreased, their enzymatic activity was not affected (24). Consistent with that study, we observed a decreased mRNA expression of *CAT* in AD patients but without statistical significance. However, since CAT enzyme activity was not measured, a comparison could not be made like the study of González-Mundo et al.

Considering the fact that the brain is the fattiest organ after adipose tissue, ACSL4 is regarded as an important enzyme for neurodegenerative diseases due to its role in lipid metabolism although it has not been fully investigated in AD (25). An *in vitro* study of embryonic stem cells showed that the nerve growth factor, retinoic acid induced neuronal differentiation, and neurite growth were weakened by a knockout of the *ACSL4* gene (26). This study showed a non-significantly decreased expression level of *ACSL4* in AD patients, also a correlation analysis pointed out that the expression of *ACSL4* decreased with increasing age of onset. The fact that AD patients had higher *ACSL4* expression levels may support the assumption that a lipid metabolism pathway is involved in the pathogenesis of oxidative stress in AD. Since our study is the first investigating the *ACSL4* expression levels in AD patients, it is necessary to replicate our findings in larger patient-control groups and to support by further *in vitro* studies.

Aging is a major risk factor for AD, and it is known that the expression of several genes are altered during aging. Many studies have shown that the expression of various genes involved in DNA repair, energy metabolism, oxidative stress response, and synaptic activity changes with aging (27). Although limited to animal studies, *GPX4* and *SLC7A11* gene expressions have been shown to alter with age, while the effect of age on *ACSL4* gene expression is unknown (28,29). Also, a study by Tatone et al. showed that the *CAT* mRNA levels were significantly lower in women over the age of 38 (30). In this study, we did not find any correlation between age and the expression of studied genes. This may suggest that age may not influence the expression of these genes, but it should not be ruled out since this research was conducted with a small study group. Although the age of the patients and controls was similar, we found a statistically significant difference between them. However, no significant effect of age was observed in correlation analysis.

Our study has several limitations. First, the current study was conducted using a small sample size. Second, the activities

of enzymes that are products of studied genes could not be measured. Therefore, the ability to investigate the relationship between extracellular enzymatic activity and mRNA expression levels was hampered. Lastly, considering AD is a CNS disease, the whole blood-based mRNA analysis is a limitation factor. Future research should focus on mRNA expression in brain tissues from Alzheimer's patients and *in vitro* cell cultures. The present study laid the groundwork for future research into oxidative stress related gene expressions in AD.

CONCLUSION

In conclusion, the results showed that peripheral blood expressions of the *GPX4, SLC7A11, CAT* and *ACSL4* genes were not altered in AD. However, further studies investigating the expression of these genes in brain tissue rather than peripheral blood will be more informative to elucidate their roles in AD etiology.

Ethics Committee Approval: The Istanbul University's Ethics Committee gave approval to the study's procedures. All participants signed informed consent forms.

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REFERENCES

1. Mantzavinos V, Alexiou A. Biomarkers for Alzheimer's Disease diagnosis. Curr Alzheimer Res 2017; 14(11): 1149-54. [CrossRef]
2. Mayeux R, Stern Y. Epidemiology of Alzheimer disease. Cold Spring Harb Perspect Med 2012; 2(8): a006239. [CrossRef]
3. Gatz M, Reynolds CA, Fratiglioni L, Johansson B, Mortimer JA, Berg S, et al. Role of genes and environments for explaining Alzheimer disease. Arch Gen Psychiatry 2006; 63(2): 168-74. [CrossRef]
4. Tanzi RE. The genetics of Alzheimer disease. Cold Spring Harb Perspect Med 2012; 2(10): a006296. [CrossRef]
5. Van Cauwenberghe C, Van Broeckhoven C, Sleegers K. The genetic landscape of Alzheimer disease: clinical implications and perspectives. Genet Med2016; 18(5): 421-30. [CrossRef]
6. Nita M, Grzybowski A. The Role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. Oxid Med Cell Longev 2016; 2016: 3164734. [CrossRef]
7. Tönnies E, Trushina E. Oxidative stress, synaptic dysfunction, and Alzheimer's Disease. J Alzheimers Dis 2017; 57(4): 1105-21. [CrossRef]
8. Chen K, Kazachkov M, Yu PH. Effect of aldehydes derived from oxidative deamination and oxidative stress on β-amyloid aggregation; pathological implications to Alzheimer's disease. J Neural Transm 2007; 114(6): 835-9. [CrossRef]
9. Mayes J, Tinker-Mill C, Kolosov O, Zhang H, Tabner BJ, Allsop D. β-Amyloid fibrils in Alzheimer Disease are not inert when bound to copper ions but can degrade hydrogen peroxide and generate reactive oxygen species. J Biol Chem 2014; 289(17): 12052-62. [CrossRef]
10. Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S, et al. The expression of key oxidative stress-handling genes in different brain regions in Alzheimer's disease. J Mol Neurosci 1998; 11(2): 151-64. [CrossRef]
11. Conrad M, Friedmann Angeli JP. Glutathione peroxidase 4 (Gpx4) and ferroptosis: what's so special about it? Mol Cell Oncol 2015; 2(3): e995047-e. [CrossRef]
12. von Ossowski I, Hausner G, Loewen PC. Molecular evolutionary analysis based on the amino acid sequence of catalase. J Mol Evol 1993; 37(1): 71-6. [CrossRef]
13. Habib LK, Lee MTC, Yang J. Inhibitors of catalase-amyloid interactions protect cells from beta-amyloid-induced oxidative stress and toxicity. J Biol Chem 2010; 285(50): 38933-43. [CrossRef]
14. Liu X, Zhang Y, Zhuang L, Olszewski K, Gan B. NADPH debt drives redox bankruptcy: SLC7A11/xCT-mediated cystine uptake as a double-edge sword in cellular redox regulation. Genes & Diseases In press 2020. [CrossRef]
15. Yan N, Zhang J-J. The Emerging roles of ferroptosis in vascular cognitive impairment. Front Neurosci 2019; 13(811). [CrossRef]
16. Bowling AC, Beal MF. Bioenergetic and oxidative stress in neurodegenerative diseases. Life Sci 1995; 56(14): 1151-71. [CrossRef]
17. Ran Q, Gu M, Van Remmen H, Strong R, Roberts JL, Richardson A. Glutathione peroxidase 4 protects cortical neurons from oxidative injury and amyloid toxicity. J Neurosci Res 2006; 84(1): 202-8. [CrossRef]
18. Hambright WS, Fonseca RS, Chen L, Na R, Ran Q. Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration. Redox Biol 2017; 12: 8-17. [CrossRef]
19. da Rocha TJ, Silva Alves M, Guisso CC, de Andrade FM, Camozzato A, de Oliveira AA, et al. Association of GPX1 and GPX4 polymorphisms with episodic memory and Alzheimer's disease. Neurosci Lett 2018; 666: 32-7. [CrossRef]
20. Bridges RJ, Natale NR, Patel SA. System xc- cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. Br J Pharmacol 2012; 165(1): 20-34. [CrossRef]

21. Qin S, Colin C, Hinners I, Gervais A, Cheret C, Mallat M. System Xc- and apolipoprotein E expressed by microglia have opposite effects on the neurotoxicity of amyloid-beta peptide 1-40. J Neurosci 2006; 26(12): 3345-56. [CrossRef]
22. Lin C-H, Lin P-P, Lin C-Y, Lin C-H, Huang C-H, Huang Y-J, et al. Decreased mRNA expression for the two subunits of system xc−, SLC3A2 and SLC7A11, in WBC in patients with schizophrenia: Evidence in support of the hypo-glutamatergic hypothesis of schizophrenia. J Psychiatr Res 2016; 72: 58-63. [CrossRef]
23. Nandi A, Yan L-J, Jana CK, Das N. Role of catalase in oxidative stress- and age-associated degenerative diseases. Oxid Med Cell Longev 2019; 2019: 9613090. [CrossRef]
24. González-Mundo I, Pérez-Vielma NM, Gómez-López M, Fleury A, Correa-Basurto J, Rosales-Hernández MC, et al. DNA methylation of the RE-1 silencing transcription factor in peripheral blood mononuclear cells and gene expression of antioxidant enzyme in patients with late-onset Alzheimer disease. Exp Gerontol 2020; 136: 110951. [CrossRef]
25. Fernandez RF, Ellis JM. Acyl-CoA synthetases as regulators of brain phospholipid acyl-chain diversity. Prostaglandins Leukot Essent Fatty Acids 2020; 161: 102175. [CrossRef]
26. Cho Y-Y. A novel role of brain-type ACS4 isotype in neuronal differentiation. Biochem Biophys Res Commun 2012; 419(3): 505-10. [CrossRef]
27. Işıldak U, Somel M, Thornton JM, Dönertaş HM. Temporal changes in the gene expression heterogeneity during brain development and aging. Sci Rep 2020; 10(1): 4080. [CrossRef]
28. Zheng Y, Ritzenthaler JD, Burke TJ, Otero J, Roman J, Watson WH. Age-dependent oxidation of extracellular cysteine/cystine redox state (Eh(Cys/CySS)) in mouse lung fibroblasts is mediated by a decline in Slc7a11 expression. Free Radic Biol Med 2018; 118: 13-22. [CrossRef]
29. Thiab NR, King N, McMillan M, Alghamdi OAS, Jones GL. Age-related protein and mRNA expression of glutathione peroxidases (GPx) and Hsp-70 in different regions of rat kidney with and without stressor. AIMS Mol Sci 2016; 3(2): 125-37. [CrossRef]
30. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, et al. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. Mol Hum Reprod 2006; 12(11): 655-60. [CrossRef]

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Glucosylceramide Synthase Is a Novel Biomarker of Midostaurin-Induced Cytotoxicity in Non-Mutant FLT3 Positive Acute Myeloid Leukemia Cells

Glukozilseramid Sentaz Mutant Olmayan FLT3 Pozitif Akut Miyeloid Lösemi Hücrelerinde Midostaurin İlişkili Sitotoksisitenin Yeni Bir Biyobelirtecidir

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ABSTRACT

Objective: Glucosylceramide (GC) synthesized by glucosylceramide synthase (GCS) favors cell survival and proliferation in many cancers. However, it's role in Fms-like tyrosine kinase 3 (FLT3) non-mutant Acute Myeloid Leukemia (AML) pathogenesis is not clarified. Midostaurin, a multi-kinase inhibitor, clinically benefits FLT3-mutated AML, however, its clinical efficacy is under-estimated in FLT3 non-mutant AML. This study aimed to investigate the efficacy of combination of midostaurin with GCS inhibitor in FLT3 AML cell carrying wild-type FLT3 and the underlying molecular mechanisms.

Material and Method: Cytotoxic and cytostatic effects of midostaurin, PDMP (GCS inhibitor) alone and in combination on THP1 cells were determined by MTT assay and flow cytometric propidium iodide (PI) staining, respectively. Calcusyn software was used to calculate combination indexes (CIs). GCS expression was checked by western blot.

Results: Midostaurin downregulated GCS. Simultaneous inhibition of FLT3 and GCS resulted in suppression of cell proliferation as compared to untreated control. Combinations showed synergistic cytotoxic effects (CI<1). Co-treatments increased cell cycle population at G2/M phase.

Conclusion: Inhibition of GCS enhances the efficacy of midostaurin in FLT3 non-mutant AML, which could be a novel therapeutic approach to increase midostaurin's limited usage in the clinic after detailed mechanistic studies.

Keywords: Cell cycle, FLT3 non-mutant AML, glucosylceramide synthase, midostaurin

ÖZ

Amaç: Glukozilseramid sentaz (GSS) tarafından sentezlenen glukozilseramid (GS) birçok kanser türünde hücre yaşamını ve proliferasyonunu sağlamaktadır. Ancak, mutant olmayan Fms-benzeri tirozin kinase 3 (FLT3) pozitif akut miyeloid lösemi (AML) patogenezindeki rolü açıklanmamıştır. Çoklu kinaz inhibitörü olan midostaurin mutant FLT3 AML tedavisinde etkili olmasına rağmen mutant olmayan FLT3 pozitif AML'deki klinik etkisi gözden kaçırılmıştır. Bu çalışmada, midostaurinin GSS inhibitörü ile kombinasyonunun yabanıl tip FLT3 ifadesine sahip AML hücrelerindeki etkisinin belirlenmesi ve moleküler mekanizmalarının açıklanması amaçlanmıştır.

Gereç ve Yöntem: Midostaurin, PDMP (GSS inhibitörü) ve kombinasyonların THP1 hücreleri üzerindeki sitotoksik ve sitostatik etkileri sırasıyla MTT testi ve PI boyaması ile akım sitometri kullanılarak belirlenmiştir. Kombinasyon indeksleri (CI) Calcusyn programı ile hesaplanmıştır. GSS ifadesi western blot ile belirlenmiştir.

Bulgular: Midostaurin GSS ifadesini baskılamıştır. FLT3 ve GSS'ın birlikte inhibe edilmesi kontrolle karşılaştırıldığında hücre çoğalmasını baskılamıştır. Kombinasyonlar sinerjistik sitotoksik etki göstermiştir (CI<1). Kombinasyon hücre döngüsünün G2/M fazındaki hücre populasyonunu arttırmıştır.

Sonuç: Mutant olmayan FLT3 AML'de GSS inhibisyonunun midostaurin'in etkisini arttırdığı saptanmıştır. Detaylı mekanizma çalışmaları yapıldıktan sonra kombinasyon tedavisinin midostaurin'in sınırlı klinik kullanımını arttırması açısından yeni bir yaklaşım olabileceği düşünülmektedir.

Anahtar Kelimeler: Hücre döngüsü, FLT3 mutant olmayan AML, glukosilseramid sentaz, midostaurin

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INTRODUCTION

Acute myeloid leukemia (AML) is a genetically complex disorder, which results in different prognostic subgroups (1). Fms-like tyrosine kinase 3 (FLT3) gene is considered as the most important one among the altered genes in AML, leading to clinically problematic FLT3 positive AML subgroups in both newly diagnosed adult patients and pediatric AML (2).

The FLT3 gene is included in the receptor tyrosine kinase family and commonly found on the plasma membrane of hematopoietic stem and early progenitor cells and over-expressed or mutated on a high percentage of AML blasts (3). Dimerization and activation of FLT3 is induced after binding of FLT3 ligand, which leads to activated downstream RAS, PI3K/AKT and STAT5 signaling pathways involved in cell proliferation and inhibition of apoptosis (3,4). FLT3 receptor and its downstream pathways become constitutively active as a result of two common FLT3 mutations, which are found in tyrosine kinase domain (TKD) and within its juxtamembrane domain (internal tandem duplication, ITD) (2,4). Therefore, development of new small molecule inhibitors has dramatically changed the treatment course of FLT3 positive AML after defining the critical role of FLT3 signaling in disease pathogenesis. FLT3 inhibitors include first generation inhibitors such as midostaurin and sorafenib and next generation inhibitors such as crenolanib and gilteritinib, which differ in their specificity and potency (5). Among these inhibitors, midostaurin (PKC412) was the first one clinically approved for the treatment of newly diagnosed FLT3-mutation carrying patients in combinations with standard treatment protocols (6). However, some clinical trials revealed that midostaurin is also effective in non-mutant FLT3 positive AML, since 70-100% of AML patients overexpress FLT3 and it has multiple cellular targets in addition to FLT3 (7,8). Molecular studies investigating this transient effect of midostaurin in non-mutant FLT3 AML are very scarce, which clearly indicated that FLT3-independent molecules or pathways might have a role to regulate its efficacy. In a study, ERK and KIT kinases have been shown to be targeted by midostaurin and their pharmacological inhibition enhanced its effect (9). In another study, MEK was identified as a midostaurin target and co-inhibition of MEK and FLT3 gave promising results (10). Therefore, we suggest that one of the mechanisms contributing to midostaurin's efficacy in non-mutant FLT3 AML could be the glucosylceramide synthase (GCS) enzyme involved in ceramide (Cer) catabolism, which has many therapeutic targets in different cancers.

Sphingolipids including Cer and its phosphorylated or glycosylated forms determine the cell fate, which is either cell survival or cell death (11). Members of the sphingolipid family affect cancer initiation and progression by modulating cell growth, and division, apoptosis, metastasis and response to therapy (12). The central molecule Cer derived is known to be an apoptotic lipid whilst its glycosylated form glucosylceramide (GC) is involved in cell proliferation and drug resistance (11,13). Cer produced via *de novo* or salvage pathway is transferred to *cis*-Golgi to be glycosylated by GSC, hence this enzyme with its anti-carcinogenic activities is shown to possess therapeutic importance in cancers (14). Inhibition of GCS in head and neck cancer overcame resistance to cisplatin by downregulating p-glycoprotein and upregulating apoptotic proteins (15). In Chronic Myeloid Leukemia (CML) cells, GCS was found overexpressed in imatinib-resistant cells as compared to sensitive partners and targeting GCS sensitized resistant cells to imatinib (16). Delivery of Cer nanoliposomes plus simultaneous inhibition of GCS resulted in induction of the intrinsic pathway of apoptosis in natural killer cell leukemia (17). Therefore, investigation of the involvement of GCS in midostaurin's effect on non-mutant FLT3 AML could open a new route to understand midostaurin's observed clinical activity and provide a novel target.

In this study, we hypothesized that inhibiting GCS in combination with midostaurin would enhance midostaurin's antileukemic activity in THP1 cells for the first time, and we could suggest a possible combination approach.

MATERIAL AND METHOD

Chemicals and Agents

MTT and midostaurin were commercially supplied (Sigma-Aldrich, USA). 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Stock solutions (10 mM in dimethyl sulfoxide (DMSO)) were prepared, which do not contain more than 0.01% DMSO in culture. RPMI 1640 growth medium and all ingredients required for complete growth medium (Penicillin-streptomycin and fetal bovine serum) were obtained from Invitrogen (Paisley, UK).

Cell Lines and Culture Conditions

THP1 cell line, representing non-mutant/wild type FLT3 carrying AML cell line, was purchased from German Collection of Microorganisms and Cell Cultures (Germany, DSMZ) and cultured and maintained in RPMI-1640 growth medium (with L-glutamine) including 10-20% fetal bovine serum (FBS) and 1% penicillin-streptomycin up to 15 passages at 37°C in a 5% CO_2 incubator.

Cell Viability Assay

Cell viability was analyzed by the standard MTT protocol in response to increasing concentrations of midostaurin (100-1000 nM), and PDMP (1-80 µM) on THP1 cells (18). $1x10^4$ cells/well were seeded in 96-well plates for 48 h. Following treatments, a 20 µl MTT solution (5 mg/mL, Sigma Aldrich) was added. Then, absorbances were recorded at 570 nm using a spectrophotometer. Based on the cell proliferation graphs, IC_{50} values (concentration inhibiting cell growth by 50%) for midostaurin and PDMP were calculated by linear regression analysis using GraphPad software (San Diego, CA).

Combination Index (CI) Analysis

Midostaurin (200-800 nM) was combined with PDMP (10-40 µM) at a fixed molar ratio. After 48 h of exposure, cell survival was assessed by the standard MTT assay. The method of Chou and Talalay was used to define whether combinations lead to synergism (CI<1), additivity (CI:1.0-1.1) or antagonism (CI>1.1) using CalcuSyn software (Biosoft, Cambridge, United Kingdom) (19,20).

Cell Cycle Analysis

7.5x10^5 cells/2ml were treated with midostaurin (400-800 nM), PDMP (20-40 µM) and their combinations (synergistic combinations were chosen based on CI analysis) for 48 h. Then, the cells were fixed with cold ethanol overnight at -20°C. The cells were treated with RNase-A (200 µg/ml, Sigma Aldrich) at 37°C for 30 min and then with propidium iodide (PI) (1 mg/ml, Sigma Aldrich) at room temperature for 10-15 minutes (18). Cell cycle analysis was carried out by BD FACSCalibur flow cytometer (BD Biosciences). The results were analyzed using BD FACSDiva™ (BD Biosciences).

Western Blot Analysis

5x10^6 cells were incubated with midostaurin (200 and 400 nM) for 48 h to detect the changes in the expression of GCS by western blot. After cell lysis in the RIPA buffer (Sigma-Aldrich, USA), protein amounts were detected with RC DC™ Protein Assay Kit (Bio-Rad, USA). 30 µg protein was loaded to SDS-PAGE and separated proteins were transferred to PVDF membranes. Primary antibodies for GCS (1:3000, Novus Biologicals, USA) and Beta Actin (1:3000, Cell Signaling, USA) were used to detect the proteins and conjugated with secondary antibodies (1:10000, Jackson ImmunoResearch, USA). The protein bands were visualized with Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific™,USA). Densitometric analysis of immunoreactive bands was carried out by the imaging software (Bio-Rad, ChemiDoc, Image LabTM 3.0).

Statistical Analysis

Three independent experiments were done where the results are expressed as mean±standard deviation (SD). GraphPad Prism 6.0 was used for statistical analysis. One-way ANOVA was applied followed by Dunnett's or Tukey's multiple comparisons tests. $p<0.05$ was accepted as statistically significant.

RESULTS

Midostaurin Decreased Glucosylceramide Synthase Level in THP1 Cells

Changes in GCS protein level in response to sub-toxic concentrations of midostaurin (200 and 400 nM) were analyzed. 400 nM midostaurin caused a 0.5-fold decrease in GCS expression compared with the untreated control group (Figure 1) although 200 nM midostaurin did not affect GCS expression significantly. Therefore, it could be suggested that GCS might be a critical enzymatic target involved in non-mutant FLT3 positive AML pathogenesis.

Co-inhibition of FLT3 and GCS Exerted Synergistic Cytotoxic Effect in THP1 Cells

It was previously shown that midostaurin alone inhibited the viability of THP1 cells with an IC_{50}: 916 nM (Figure 2A) (21). PDMP alone did not affect leukemic cell viability significantly up to 40 µM and its IC_{50} was approximately 63 µM (Figure 2B). To determine whether the inhibition of GCS by PDMP could affect anti-proliferative action of midostaurin, THP1 cells were treated with midostaurin plus PDMP by using their sub-toxic concentrations for 48 h. As shown in Figure 2C, at midostaurin concentrations of 400 nM and 800 nM, viability of THP1 cells exposed to 20 and 40 µM PDMP was significantly inhibited as compared to the control. (Figure 2C). Viability of the cells treated with 800 nM midostaurin in combination with 40 µM PDMP was significantly lower than that of the cells treated with 800 nM midostaurin alone (Figure 2C). CIs were also calculated (Figure 2D). The combination of 400 and 800 nM midostaurin with 20 and 40 µM PDMP, respectively resulted in synergistic toxicity (CIs: 0.6 and 0.8)

Co-targeting FLT3 and GCS Arrested THP1 Cells at G2/M Phase

To determine whether the enhanced midostaurin toxicity in the presence of PDMP was related to changes in distribution of cell cycle populations, we analyzed the cell cycle percentages of the cells treated with midostaurin and PDMP alone or their combinations based on CalcuSyn analysis. 400 and 800 nM midostaurin alone blocked cell cycle progression at the G2/M phase (20.1 and 36.4 vs 9.5%, respectively) as shown in the previous study (21), whereas PDMP alone did not induce changes in any of the cell cycle phases as compared to control (Figure 3A and Figure 3B). Both combinations stopped the cell cycle at the G2/M phase (37 and 37.1 vs 9.5%, respectively) as

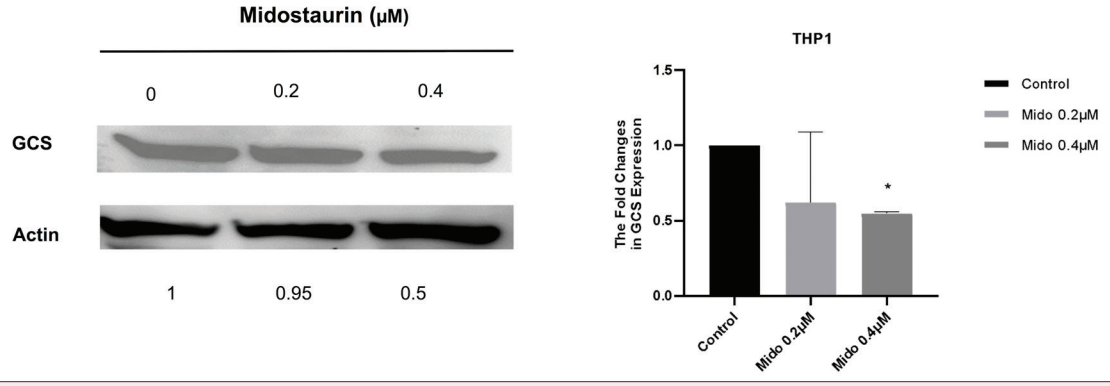
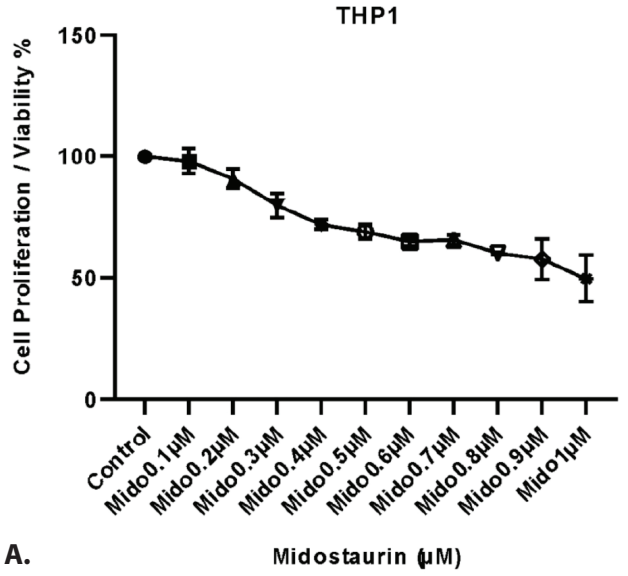
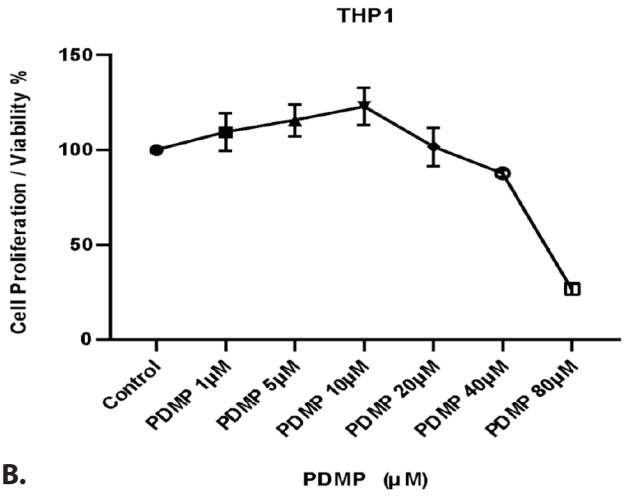
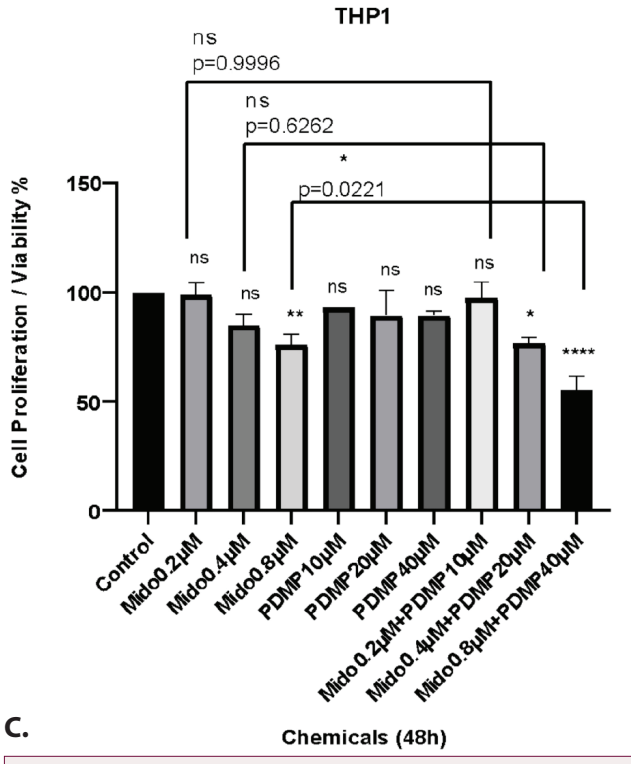
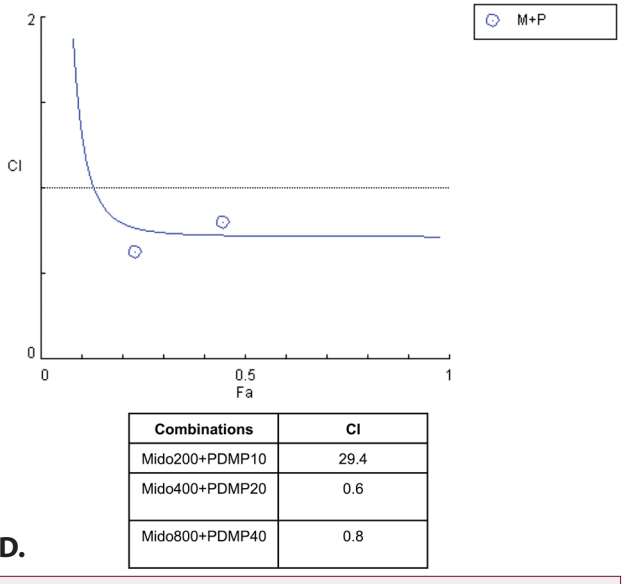


Figure 1. Western blot analysis of GCS expression in response to midostaurin in THP1 cells. The results from three independent experiments were given as mean ± SD. *p<0.05 vs. control. GCS: Glucosylcermide synthase; Mido: Midostaurin









Combinations	CI
Mido200+PDMP10	29.4
Mido400+PDMP20	0.6
Mido800+PDMP40	0.8

Figure 2. Anti-proliferative effects of midostaurin (A), PDMP (B) and combinations (C) in THP1 cells. Results are shown as means ± SD of three experimental setups performed at different times. Calculation of CI values based on the method of Chou and Talalay (D).*p<0.05, **p<0.005, ****p<0.0001 versus control. Mido: Midostaurin

compared to control. 400 nM midostaurin with 20 µM PDMP arrested the cells at G2/M phase as compared to midostaurin alone (20.1% and 37%, respectively) (Figure 3A and Figure 3B). These data further supported the enhancing effect of GCS inhibition on the cytotoxicity of midostaurin alone.

DISCUSSION

The roles of sphingolipid metabolism's intermediates including Cer and its modified forms in carcinogenesis and response to therapy have been extensively investigated (22). In this concept, we explored the role of GCS for the first time in mi-

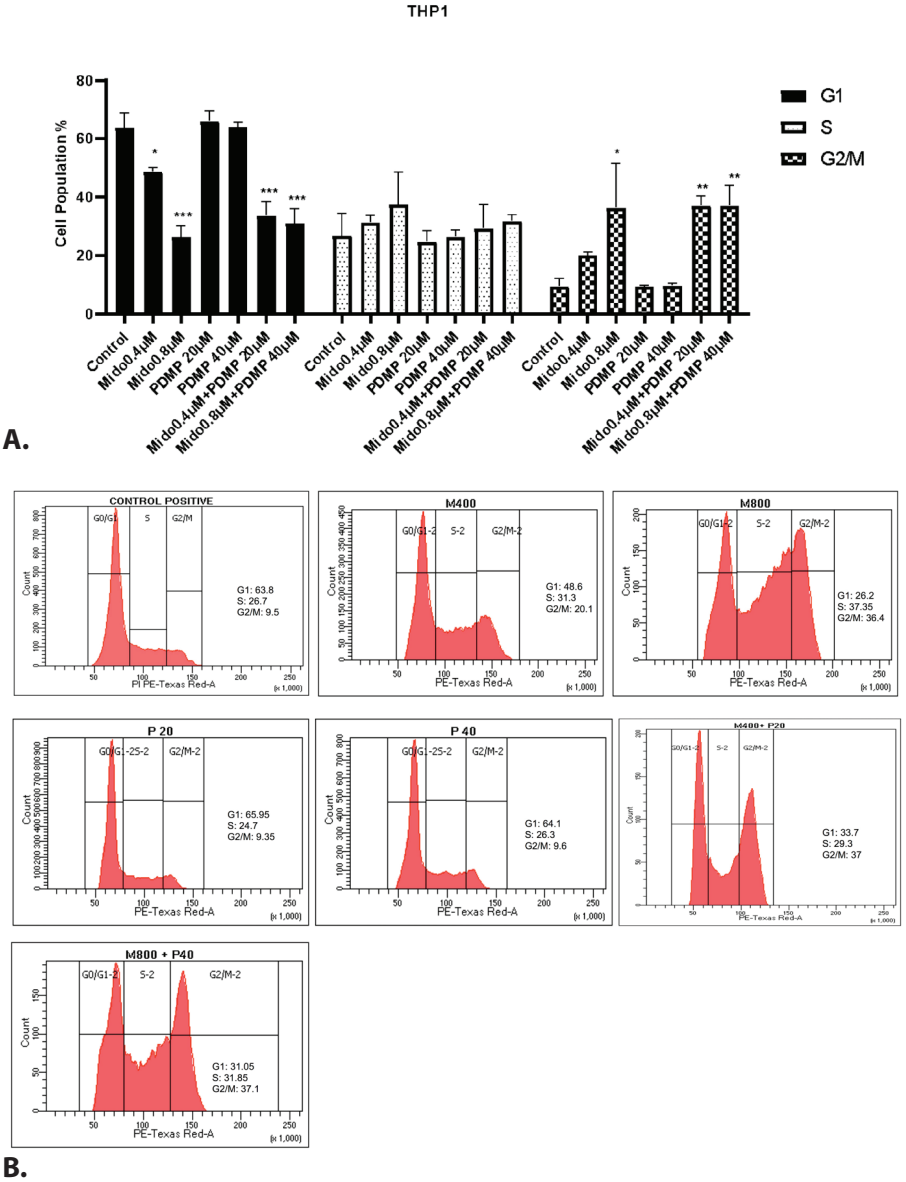


Figure 3. Cell cycle distribution of THP1 cells treated with midostaurin, PDMP and combinations. Data are shown as the means ± SD of three experimental setups performed at different times. *p<0.05, **p<0.005, ***p<0.0005 versus control. Mido: Midostaurin

dostaurin's anti-leukemic action in non-mutant FLT3 positive AML although its clinical approval has been granted for only patients carrying FLT3 mutations (23). Midostaurin's activity in non-mutant FLT3 AML patients was also encouraging based on a phase III trial (24), which could suggest the presence of FLT3-independent targets responsible for its broad activity as explained previously (9,10).

GCS resides in golgi apparatus and catalyzes the conversion of Cer into GC by transferring UDP-glucose to Cer. GC is shown to be overexpressed in various cancers and found to be associated with increased cell proliferation and failed treatment response (14). In this study, we suggest that GCS could be associated with observed clinical response in phase studies and a new therapeutic target could be defined if its role in midostaurin's action is enlightened in non-mutant FLT3 positive AML.

The protein level of GCS was decreased after midostaurin treatment as compared to untreated cells (Figure 1) and untreated cells had also high GCS expression, suggesting that GCS could be involved in response to midostaurin treatment as well as having a role in non-mutant FLT3 positive AML pathogenesis. There are studies regarding the role of GCS in several hematological malignancies. In resistant chronic myeloid leukemias,

GCS was shown to be upregulated (16,25). Based on this result, THP1 cells were treated with midostaurin plus PDMP to determine the presence of any synergistic cytotoxic effect. The results suggested that midostaurin's growth inhibitory effect on THP1 cells was enhanced via showing synergistic inhibition of cell viability (CIs<1 for combinations) (Figure 2C and Figure 2D). Hence, GCS could be defined as a therapeutic target in THP cells, which is in accordance with the findings in several cancers. Inhibition of GCS by using a specific inhibitor together with chemotherapeutics sensitized resistant leukemia cells by increasing apoptosis (25). In prostate cancer cells, co-treatment with docetaxel and GCS inhibitor enhanced docetaxel's cytotoxic activity by inducing strong synergism (26). PDMP also potentiated the cytotoxic effect of dasatinib in CML cells with CI values lower than 1 (27).

To further investigate the enhanced cytotoxicity of midostaurin induced by PDMP, cell cycle populations were quantified after treatment with midostaurin and PMDP alone and in combinations. The results showed that PDMP alone did not cause changes at cell cycle phases, however midostaurin alone arrested the cells at G2/M (Figure 3). This cytostatic effect of midostaurin was in accordance with studies comparing the effects of midostaurin on cell cycle distribution between non-mutant and mutant FLT3 positive AML and FLT3 wild type AML (28,21). The studies related to cell cycle profiles of the leukemic cells treated with GCS inhibitors showed varying results based on the leukemia type. In K562 CML cells, PDMP exposure resulted in G1 arrest and it caused more accumulation together with imatinib (16). Another GCS inhibitor PPPP did not change cell cycle profiles of leukemic cells alone, however, combination with vincristine increased G2/M population (29). In this study, co-treatments induced G2/M arrest as compared to the control or midostaurin alone (Figure 3). There are very limited studies investigating the contribution of Cer or its metabolites to midostaurin's effect in cancer in which liposomal Cer treatment or sphingosine kinase-1 inhibition increased midostaurin's activity (30,21).

In conclusion, midostaurin's cytotoxic activity is enhanced in the presence of PDMP, which supported the involvement of GCS in midostaurin treatment and non-mutant FLT3 positive AML pathogenesis through inducing synergistic cell cycle arrest. Even though this preliminary study could suggest a new target of midostaurin, it is still needed to elucidate the relationship between midostaurin and GCS mechanistically. It would be suggested to investigate the role of signaling pathways leading to cell death and to analyze cell cycle regulators involved in G2/M phase regulation in response to co-treatments. Pre-clinical *in vitro* models, *in vivo* mouse studies and *ex vivo* patient samples could be studied to reveal the expression level of GCS after midostaurin treatment.

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REFERENCES

1. Blum WG, Mims AS. Treating acute myeloid leukemia in the modern era: A primer. Cancer 2020; 126(21): 4668-77. [CrossRef]
2. Ambinder AJ, Levis M. Potential targeting of FLT3 acute myeloid leukemia. Haematologica 2021; 106(3): 671-81. [CrossRef]
3. Kennedy VE, Smith CC. FLT3 Mutations in acute myeloid leukemia: Key concepts and emerging controversies. Front Oncol 2020; 10: 612880. [CrossRef]
4. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia 2019; 33: 299-312. [CrossRef]
5. Antar AI, Otrock ZK, Jabbour E, Mohty M, Bazarbachi A. FLT3 inhibitors in acute myeloid leukemia: ten frequently asked questions. Leukemia 2020; 34(3): 682-96. [CrossRef]
6. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. N Eng J Med 2017; 377(5): 454-64. [CrossRef]
7. Fischer T, Stone RM, Deangelo DJ, Galinsky I, Estey E, et al. Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. J Clin Oncol 2010; 28(28): 4339-45. [CrossRef]
8. Stone RM, Fischer T, Paquette R, Schiller G, Schiffer CA, et al. Phase IB study of the FLT3 kinase inhibitor midostaurin with chemotherapy in younger newly diagnosed adult patients with acute myeloid leukemia. Leukemia 2012; 26(9): 2061-8. [CrossRef]

9. Weisberg E, Meng C, Case AE, Tiv HL, Gokhale PC et al. Effects of the multi-kinase inhibitor midostaurin in combination with chemotherapy in models of acute myeloid leukaemia. J Cell Mol Med 2020; 24(5): 2968-80. [CrossRef]
10. Morales ML, Arenas A, Ortiz-Ruiz A, Leivas A, Rapado I, et al. MEK inhibition enhances the response to tyrosine kinase inhibitors in acute myeloid leukemia. Sci Rep 2019; 9(1): 18630. [CrossRef]
11. Giussani P, Tringali C, Riboni L, Viani P, Venerando B. Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. Int J Mol Sci 2014; 15: 4356-92. [CrossRef]
12. Truman JP, Garcia-Barros M, Obeid LM, Hannun YA. Evolving concepts in cancer therapy through targeting sphingolipid metabolism. Biochim Biophys Acta 2014; 1841: 1174-88. [CrossRef]
13. Morad SA, Cabot MC. Ceramide-orchestrated signalling in cancer cells. Nat Rev Cancer 2012; 13: 51-65. [CrossRef]
14. Kartal Yandım M, Apohan E, Baran Y. Therapeutic potential of targeting ceramide/glucosylceramide pathway in cancer. Cancer Chemother Pharmacol 2013; 71(1): 13-20. [CrossRef]
15. Roh JL, Kim EH, Park JY, Kim JW. Inhibition of Glucosylceramide Synthase Sensitizes Head and Neck Cancer to Cisplatin. Mol Cancer Ther 2015; 14(8): 1907-15. [CrossRef]
16. Baran Y, Bielawski J, Gunduz U, Ogretmen B. Targeting glucosylceramide synthase sensitizes imatinib-resistant chronic myeloid leukemia cells via endogenous ceramide accumulation. Cancer Res Clin Oncol 2011; 137(10): 1535-44. [CrossRef]
17. Watters RJ, Fox TE, Tan SF, Shanmugavelandy S, Choby JE, et al. Targeting glucosylceramide synthase synergizes with C6-ceramide nanoliposomes to induce apoptosis in natural killer cell leukemia. Leuk Lymphoma 2013; 54(6): 1288-96. [CrossRef]
18. Adan A, Baran Y. The pleiotropic effects of fisetin and hesperetin on human acute promyelocytic leukemia cells are mediated through apoptosis, cell cycle arrest, and alterations in signaling networks. Tumor Biology 2015; 36(11): 8973-84. [CrossRef]
19. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 2010; 70: 440-6. [CrossRef]
20. Casagrande N, Borghese C, Favero A, Vicenzetto C, Aldinucci D. Trabectedin overcomes doxorubicin-resistance, counteracts tumor-immunosuppressive reprogramming of monocytes and decreases xenograft growth in Hodgkin lymphoma. Cancer Lett 2021; 500: 182-93. [CrossRef]
21. Sahin HN, Adan A. Combinatorial effect of midostaurin and sphingosine kinase-1 inhibitor on FLT3 wild type acute myeloid leukemia cells. Turkish Journal of Biochemistry 2021; in press
22. Kroll A, Cho HE, Kang MH. Antineoplastic agents targeting targeting sphingolipid pathways. Front Oncol 2020; 10: 833. [CrossRef]
23. Levis M. Midostaurin approved for FLT3-mutated AML. Blood 2017; 129(26): 3403-6. [CrossRef]
24. Dohner H, Sierra J, Stone R, Hoenekopp A, Berkowitz N, et al. Trial in progress: A phase 3, randomized, double-blind study of midostaurin in combination with chemotherapy and as single-agent maintenance therapy in newly diagnosed patients with FLT3 mutation-negative acute myeloid leukemia (AML). Clin Lymphoma Myeloma Leuk 2018; 18(Suppl 1): S206-7. [CrossRef]
25. Salustiano EJ, da Costa KM, Freire-de-Lima L, Mendonça-Previato L, Previato JO. Inhibition of glycosphingolipid biosynthesis reverts multidrug resistance by differentially modulating ABC transporters in chronic myeloid leukemias. J Biol Chem 2020; 295(19): 6457-71. [CrossRef]
26. Bassoy EY, Baran Y. Bioactive sphingolipids in docetaxel-induced apoptosis in human prostate cancer cells. Biomed Pharmacother 2012; 66(2): 103-10. [CrossRef]
27. Gencer EB, Ural AU, Avcu F, Baran Y. A novel mechanism of dasatinib-induced apoptosis in chronic myeloid leukemia; ceramide synthase and ceramide clearance genes. Ann Hematol 2011; 90(11): 1265-75. [CrossRef]
28. Odgerel T, Kikuchi J, Wada T, Shimizu R, Futaki K, et al. The FLT3 inhibitor PKC412 exerts differential cell cycle effects on leukemic cells depending on the presence of FLT3 mutations. Oncogene 2008; 27(22): 3102-10. [CrossRef]
29. Olshefski RS, Ladisch S. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. Int J Cancer 2001; 93(1): 131-8. [CrossRef]
30. Zhu Y, Wang C, Zhou Y, Ma N, Zhou J. C6 ceramide motivates the anticancer sensibility induced by PKC412 in preclinical head and neck squamous cell carcinoma models. J Cell Physiol 2018; 233(12): 9437-46. [CrossRef]

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Histone Deacetylase, Xanthine Oxidase and Urease Inhibitory Activities of *Eremurus spectabilis* M. Bieb. Extracts

Eremurus spectabilis Bieb. Ekstrelerinin Histon Deasetilaz, Ksantin Oksidaz ve Üreaz İnhibitör Aktiviteleri

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ABSTRACT

Objective: Natural ingredients derived from plants (especially edible ones) continue to be investigated for understanding whether they have therapeutic/preventive effects on certain ailments, such as cancer and gastrointestinal disorders. The main goal of the current work is to investigate the inhibitory potential of *Eremurus (E) spectabilis* M. Bieb. extracts on histone deacetylase (HDAC), xanthine oxidase (XO) and urease activities.

Material and Method: Fresh *E. spectabilis* leaves were obtained from a local market in Eyup/Istanbul. Inhibitory activities of ethanolic and ethyl acetate extracts on HDAC, XO and urease were examined.

Results: According to our findings, ethanolic and ethyl acetate extracts of *E. spectabilis* both have revealed apparent inhibitory effects against all examined enzymes. Ethanolic extract showed a better inhibitory effect on HDAC than ethyl acetate extract. On the other hand, the inhibitory effects of ethyl acetate extract against both XO and urease were higher than the ethanolic extract.

Conclusion: Consequently, due to the inhibitory effects of the plant being studied, it might be suggested that it can be used for medicinal purposes, in raw extract form, or as a source of bioactive compounds.

Keywords: *Eremurus spectabilis*, histone deacetylase, xanthine oxidase, urease, inhibitory effect

ÖZ

Amaç: Bitkilerden elde edilen doğal bileşiklerin (özellikle yenilebilir olanlar), kanser ve gastrointestinal bozukluklar gibi belirli rahatsızlıklar üzerinde tedavi edici/önleyici etkilerinin olup olmadığı araştırılmaya devam etmektedir. Çalışmanın temel amacı, *Eremurus (E) spectabilis* M. Bieb. ekstrelerinin histon deasetilaz (HDAC), ksantin oksidaz (XO) ve üreaz aktiviteleri üzerindeki inhibitör etkilerini araştırmaktır.

Gereç ve Yöntem: *E. spectabilis*'in taze yaprakları, Eyüp/İstanbul'daki, yerel bir pazardan temin edilmiştir. Etanol ve etil asetat ekstrelerinin HDAC, XO ve üreaz üzerindeki inhibitör aktiviteleri incelenmiştir.

Bulgular: Elde edilen sonuçlara göre, *E. spectabilis*'in hem etanol hem de etil asetat ekstrelerinin incelenen tüm enzimler üzerinde belirgin inhibitör etkiye sahip olduğu bulunmuştur. Etanol ekstresi, HDAC üzerinde etil asetat ekstresinden daha iyi bir inhibisyon etkisi göstermiştir. Etil asetat ekstresinin ise, hem XO hem de üreaza karşı inhibisyon etkisinin, etanol ekstresinden daha yüksek olduğu görülmüştür.

Sonuç: Sonuç olarak, incelenen bitkinin gösterdiği inhibitör etkiler nedeni ile, bu bitkinin tıbbi amaçla, ham ekstre şeklinde veya biyoaktif bileşik kaynağı olarak kullanılabileceği ileri sürülebilir.

Anahtar Kelimeler: *Eremurus spectabilis*, histon deasetilaz, ksantin oksidaz, üreaz, inhibitör etki

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INTRODUCTION

Plants have been used as beneficial healing options to modern medicine, as well as being consumed by humans as food for many years. The investigation of the utility of herbs in the treatment of diseases becomes important, due to the possible side effects and considerable high cost of synthetic drugs (1). The genus Eremurus (Xanthorrhoeaceae) comprises 62 species and is widespread in the mountains of Central Asia and the Middle East (including Turkey) (2,3). Two Eremurus species are grown naturally in Turkey. One of them is *Eremurus (E) cappadocicus* J. Gay ex Baker and the other *E. spectabilis* which is named "çiriş otu, yabani pırasa, or güllük" between individuals (3). *E. spectabilis* grows naturally in some provinces of the Eastern Anatolian region of Turkey (4-6). In these regions, the plant is commonly used as not only a wild edible plant, but also as a medicinal plant whose leaves and roots are traditionally used to cure some disorders including diabetes, antihypertension, and eczema (7-9).

Naturally occurring secondary metabolites are found in vegetables and fruits include phenolic acids, flavonoids, and their derivatives. These compounds have a wide range of therapeutic effects (10). Medicinal plants such as *E. spectabilis* are good sources of these molecules. This is why they are frequently used as a potential inhibitor of enzymes (such as histone deacetylase (HDAC), xanthine oxidase (XO) and urease) which may play key roles in the overproduction of free radicals, thereby subsequently precipitating oxidative stress, cancer and gastrointestinal disorders (11).

HDAC is an enzyme responsible for removing acetyl groups from N-terminal N^{ε}-lysine residues of histone. In contrast, acetylation of histone is catalyzed by histone acetyltransferase. The acetylation and deacetylation of histones are strictly well balanced, this is because acetylation/deacetylation process is an important post-translational modification of histone. When an imbalance of the activities of these enzymes favors HDAC, deacetylation of histone brings about stronger histone-DNA interactions-a key factor for the regulation of mammalian gene expression. In addition, aberrant HDAC activity and/or expression of HDAC can lead to cancer induction/progression (12,13).

Xanthine oxidoreductase (XOR) is an enzyme with multiple activities as follows: Xanthine dehydrogenase (XDH), nitrite reductase, NADH oxidase and xanthine oxidase (XO). Owing to its multiple enzymatic activities, XOR has crucial physiological functions. Notably, increased XO activity is accompanied by an overproduction of uric acid, and thus hyperuricemia (14). On the other hand, the increased activity of XO causes the formation of free radicals (both oxygen-centered and nitrogen-centered reactive species) that can degenerate or promote cancer development. More so, elevated levels of plasma XO activities in patients with certain disease conditions (e.g. cancer, metabolic disorder, and diabetes) have been reported (15). From this point of view, scientists have made great efforts to find new molecules with fewer side effects than the currently used XO inhibitors.

Urease belongs to a class of hydrolases enzyme that catalyzes the hydrolyzation of urea into ammonium ion, thereafter carbamate and subsequently converted into carbon dioxide. An apparent rise in urease activity as a consequence of high pH levels in human brings about various pathophysiological conditions such as gastrointestinal disorders, peptic ulcers, and gastric cancer as well. In these cases, ureases are accepted as a key reason for developing the aforementioned disorders owing to *Helicobacter pylori* infection (16,17).

There are insufficient reports about the effects of *E. spectabilis* inhibitory activities against HDAC, XO and urease enzymes. Therefore, the main goal of this work was to determine the inhibition activities of ethanolic and ethyl acetate extracts of *E. spectabilis* against the HDAC, XO and urease enzymes.

MATERIAL AND METHOD

Plant Material and Chemicals

Fresh *E. spectabilis* leaves were obtained from a local market in Eyup/Istanbul, Turkey. The leaves were washed with distilled water and dried at room temperature. The dried plant was stored at −20 °C until required for use. The plant was inspected by Prof. Dr. Emine Akalin (Faculty of Pharmacy, Istanbul University). Also, it was registered at the Istanbul University Faculty of Pharmacy Herbarium (ISTE) with the number 93132.

The HDAC (fluorometric assay kit, CS1010-1KT), XO (from bovine milk), and urease (from jack beans) and their respective substrates were supplied from Sigma-Aldrich (St. Louis, MO). All of the chemicals used were of analytical grade.

Preparation of Extracts

The ethanolic and ethyl acetate extracts were prepared by refluxing 20 g of dried *E. spectabilis* leaves with either ethanolic or ethyl acetate for 2 hours in Soxhlet apparatus. The extracts were then filtered at room temperature, the solvents were evaporated under reduced pressure and controlled temperature (40–50 °C). The ethanolic (87.32 mg/g dry weight) and ethyl acetate (87.84 mg/g dry weight) extracts were obtained from *E. spectabilis* and they were kept at −20 °C prior to analysis.

In vitro HDAC Inhibitory Activity

HDAC inhibitory activity was evaluated based on the two-step enzymatic reaction. This was performed by measuring fluorescence in a fluorimeter plate reader as prescribed by the commercial assay kit. Valproic acid (VPA) was used as a standard. The results were expressed as the average of triplicate trials.

The percent inhibition of the HDAC was calculated using the following equation:

$$\text{HDAC inhibitory activity (\%)} = [(A-B)/A] \times 100$$

where A represents the activity of the enzyme without an inhibitor, and B is the activity of enzyme in the presence of the extract (or standard inhibitor).

In vitro XO Inhibitory Activity

XO inhibitory activity was carried out by the spectrophotometric method of Abdullahi *et al*. (18). In brief, the reaction medium comprising of 0.1 M phosphate buffer, (pH 7.50), a sample at varying concentrations, and XO solution (0.1 U/mL, in the same buffer) was mixed and allowed to stand at 25°C for 15 min. Xanthine solution (150 µM) was pipetted to the reaction medium and then incubated at 25°C for 15 min. Thereafter 1 N HCl was added to the mixture. The absorbance of the samples was recorded at 410 nm. Allopurinol was used as a standard inhibitor. The results were expressed as the average of triplicate trials.

The percent inhibition of the XO was calculated using the following equation:

XO inhibitory activity (%) = [(*A*-*B*)/*A*] x 100

where *A* represents the activity of the enzyme without an inhibitor, and *B* is the activity of enzyme in the presence of the extract (or standard inhibitor).

In vitro Urease Inhibitory Activity

Urease inhibitory activity was assayed by the spectrophotometric method developed by Van Slyke and Archibald (19), in which urea is hydrolyzed over time to yield ammonia and carbon dioxide. Briefly, an assay mixture of a total volume of 1.9 mL, containing extract solution (or standard inhibitor), phosphate buffer (100 mM, pH 6.8 and containing 500 mM urea), and urease solution (16 mg/mL) was pre-incubated at room temperature for 15 min. The reaction solution was mixed with appropriate volume of phenol-red solution, thereafter incubated at room temperature for 15 min again. After incubation, the absorbance was measured at 570 nm. Hydroxyurea was used as a standard inhibitor. The results were expressed as the average of triplicate trials.

The percent inhibition of the urease was calculated using the following equation:

Urease inhibitory activity (%) = [(*A*-*B*)/*A*] x 100

where *A* represents the activity of the enzyme without an inhibitor, and *B* is the activity of enzyme in the presence of the extract (or standard inhibitor).

For HDAC, XO, and urease inhibitory activities, the extract (or standard) concentration providing half maximum (50%) inhibitions (IC_{50}) was calculated by regression equations (by plotting extract solution concentration versus percentage inhibition). Low IC_{50} indicates the higher inhibitory potential of the tested plant extracts.

RESULTS

The inhibition effects of ethanolic and ethyl acetate extracts of *E. spectabilis* as well as that of VPA against HDAC activity are summarized in Table 1. According to the results, ethanolic extract, ethyl acetate extracts and the standard had an IC_{50} value in the range of 0.99-101.83 µg/mL. In addition, ethanolic and ethyl acetate extracts inhibited HDAC in a dose-dependent manner with IC_{50} values of 0.99±0.32 and 8.60±0.49 µg/mL, respectively. Considering the high inhibitory activities (associated with the lower IC_{50} values) these extracts demonstrated higher inhibitory activity against HDAC in comparison to VPA (IC_{50} values of 101.83±1.43 µg/mL). The order for inhibitory effect is as follows: ethanolic extract > ethyl acetate extract > VPA (Table 1).

The inhibitory activities and IC_{50} values of ethanolic extracts, ethyl acetate extracts, and allopurinol on XO are given in Table 2. It was found that the XO inhibition increased in a concentration-dependent manner for all the extracts and the standard. At 500 µg/mL of both extracts, it was observed that the ethyl acetate extract had approximately 1.5 times more inhibition value (46.16±2.34%) than ethanolic extract (30.18±1.00%). Meanwhile, at the same concentration, the inhibition value of the ethyl acetate extract was very close to that of the standard inhibitor, allopurinol (52.34±1.37%). However, the ethyl acetate extract had a lower IC_{50} value than that of ethanolic extract. On the other hand, the allopurinol had the lowest IC_{50} value (403.87±18.32 µg/mL) in comparison to both ethyl acetate

Table 1. HDAC inhibitory activity of *E. spectabilis* extracts.

Extracts/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Ethanolic Extract	0.05	5.05±0.35	0.99±0.32
	0.10	13.09±3.08	
	0.50	28.84±7.69	
Ethyl Acetate Extract	1.0	9.64±4.51	8.60±0.49
	2.0	18.48±2.32	
	5.0	31.24±0.73	
Valproic Acid	5.0	8.08±4.04	101.83±1.43
	20.0	14.82±2.50	
	50.0	27.58±1.86	

*Mean±SD; SD: Standard deviation; IC_{50}: Concentration providing half maximum (50%) inhibition

(524.13±8.17 µg/mL) and the ethanolic extracts (658.89±5.55 µg/mL) of *E. spectabilis*. The XO inhibitory activity of *E. spectabilis* extracts and the standard decreased in the order of: allopurinol > ethyl acetate extract > ethanolic extract (Table 2).

The inhibitory activities and IC_{50} values of the ethanolic, ethyl acetate extracts, and hydroxyurea on urease are presented in Table 3. All of the extracts were found to exhibit urease inhibitory activities. A comparison indicates that ethyl acetate extract had approximately 5 times more inhibitory activity than the ethanolic extract. The IC_{50} value of hydroxyurea (2422.50±102.43 µg/mL) (used as standard inhibitor) was found to be much higher than that of both the ethanolic (269.12±14.19 µg/mL) and the ethyl acetate extracts (47.15±1.63 µg/mL), respectively (Table 3). The urease inhibitory activity of *E. spectabilis* extracts and the standard decreased in the order of: ethyl acetate extract > ethanolic extract > hydroxyurea (Table 3).

Table 2. XO inhibitory activity of *E. spectabilis* extracts.

Extracts/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Ethanolic Extract	100	9.26±2.13	658.89±5.55
	250	18.44±2.26	
	500	30.18±1.00	
	750	53.46±1.00	
	1000	83.03±1.81	
Ethyl Acetate Extract	100	9.62±0.74	524.13±8.17
	250	23.72±2.46	
	500	46.16±2.34	
	750	80.45±1.61	
	1000	88.14±1.23	
Allopurinol	0.1	5.26±0.93	403.87±18.32
	1	21.78±2.85	
	10	40.65±2.08	
	100	47.47±1.14	
	500	52.34±1.37	

*Mean±SD; SD: Standard deviation; IC_{50}: Concentration providing half maximum (50%) inhibition

Table 3. Urease inhibitory activity of *E. spectabilis* extracts.

Extracts/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Ethanolic Extract	5	16.97±2.78	269.12±14.19
	10	24.24±1.05	
	50	39.39±2.78	
	100	47.88±2.78	
	500	66.06±2.77	
Ethyl Acetate Extract	1	21.24±1.72	47.15±1.63
	5	30.11±1.44	
	10	35.90±2.16	
	50	53.07±1.86	
	100	65.82±1.87	
Hydroxyurea	250	12.79±1.34	2422.50±102.43
	500	25.62±2.72	
	1000	37.21±2.68	
	2500	55.81±1.90	
	5000	80.81±3.49	

*Mean±SD; SD: Standard deviation; IC_{50}: Concentration providing half maximum (50%) inhibition

DISCUSSION

A literature search indicates that there are insufficient published reports about the effects of *E. spectabilis* inhibitory activities against HDAC, XO and urease. This encouraged us to undertake an experiment on the inhibition activities of ethanolic and ethyl acetate extracts of *E. spectabilis* against these enzymes.

HDAC activities/expressions have been shown to be elevated in cancer patients. More so, overactivity of this enzyme has been associated not only with cancer progression (due to enhanced cell proliferation), but also to cancer induction. Therefore, inhibition of HDAC is at the center of research and paramount for the discovery of new and safe molecules with anticancer properties (20). In our study, both ethanolic and ethyl acetate extracts of *E. spectabilis* were found to have much higher HDAC inhibitory activity than the VPA. On the other hand, it was reported that the anti-radical activity of ethyl acetate extract of *E. spectabilis* was better than the methanolic extract, whereas the phenolic content was higher in the methanolic extract compared to the ethyl acetate extract (21). Plant-based flavonoids are well known secondary metabolites that having a wide variety of chemical constituents, thus strong antioxidant properties (mostly due to their polyphenolic structure). Owing to the capacity of these molecules to block the distortion/diverse effects of crucial pathways and cellular signalling (especially in cancer cells), the molecules are potential and promising anticarcinogenic agent (13,22). It was revealed that *E. spectabilis* is a rich source of isoorientin (a flavonoid compound), a molecule which affected cell proliferation of neuroblastoma cells via cell cycle control (23). In the current study, the HDAC inhibitory activities of both extracts may be associated with the high amounts of phenolic compounds found in *E. spectabilis* (24). It might also be due to the involvement of other secondary metabolites of the medicinal plant.

XO is a key rate-limiting enzyme involved in purine metabolism. Increased plasma XO activity is associated with excessive synthesis of uric acid. This predominantly results in hyperuricemia, excessive formation of free radicals and subsequent triggering cancer (25,26). In this case, the most important strategy may be to find out both safe and new molecules for the inhibition of XO. According to the outcomes of our study, ethyl acetate extract exhibited a stronger XO inhibitory activity than the ethanolic extract. Moreover, it was found that ethyl acetate extract exhibited similar effects to allopurinol. *E. spectabilis* is reported to contain flavonoids such as rutin and quercetin (27). Thus, both ethanolic and ethyl acetate extracts inhibited XO activity possibly due to its rutin and quercetin content of *E. spectabilis*. In addition, quercetin may have contributed to the inhibitory effect by forming a chelate-complex with molybdenum in the active center of XO, hence resulting in a loss of activity (14,28).

The overproduction of ammonia from urea, as a consequence of high urease activity leads to an increase in pH value. This promotes excellent conditions for *Helicobacter pylori* whose overgrowth comes true, hence the progression of some stomach ailments (for example peptic ulcers, gastritis, arthritis and possibly gastric cancer) (29). Herein, the examined extracts exhibited a notable inhibitory activity against urease, with ethyl acetate extract of *E. spectabilis* having a higher effect. The effective inhibitory activity of ethyl acetate extract can be attributed to the high content of phenolics and flavonoids of the Eremurus species (30,31). Meanwhile, it had been reported that phenolic compounds and flavonoids naturally occurring in fruits/edible plants such as the Eremurus species (rather than only synthetic compounds) are promising novel and potent inhibitors against urease (32).

The limitation of the study is that the *in vitro* effects of *E. spectabilis* extract on enzymes were considered, and since many disease models are required for *in vivo* effect.

CONCLUSION

According to the present findings, ethanolic and ethyl acetate extracts of *E. spectabilis* had apparent inhibitory activities against all examined enzymes. Meanwhile, the ethanolic extract showed a better inhibitory effect on HDAC than the ethyl acetate extract. On the other hand, the inhibitory activities of the ethyl acetate extract against both the XO and urease were higher than that of the ethanolic extract. The inhibitory effects exerted by this plant make E. spectabilis an interesting candidate for medicinal use, either in the form of crude extracts or as a source of bioactive compounds.

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

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Author Contributions: Conception/Design of Study - B.B.B., R.Y.; Data Collection and/or Processing - B.B.B., R.Y.; Analysis and/or Interpretation - B.B.B., R.Y.; Writing - B.B.B., R.Y.

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REFERENCES

1. Yuan H, Ma Q, Ye L, Piao G. The traditional medicine and modern medicine from natural products. Molecules 2016; 21(5): 559. [CrossRef]
2. The genus Eremurus. (2021, May 03). Available from: http://www.theplantlist.org/tpl1.1/search?q=Eremurus
3. Salehi B, Ayatollahi SA, Segura-Carretero A, Kobarfard F, Contreras MDM, Faizi M, et al. Bioactive chemical compounds in Eremurus persicus (Joub. & Spach) Boiss. essential oil and their health implications. Cell Mol Biol 2017; 63(9): 1-7. [CrossRef]
4. Cinar A, Ay ST, Ayas, F, Karabak S, Guzelsoy N, Ucurum O. Foxtail lilly (Eremurus spectabilis M. Bieb.) as priority species biodiversity for food and nutrition project of Turkey. Anadolu J Aegean Agric Res Inst 2017; 27(2): 69-73.
5. Aysu T, Demirbaş A, Bengü AŞ, Küçük MM. Evaluation of Eremurus spectabilis for production of bio-oils with supercritical solvents. Process Saf Environ Prot 2015; 94: 339-49. [CrossRef]
6. Tosun M, Ercisli S, Ozer H, Turan M, Polat T, Ozturk E, et al. Chemical composition and antioxidant activity of foxtail lily (Eremurus spectabilis). Acta Sci Pol Hortorum Cultus 2012; 11(3): 145-53.
7. Gaggeri R, Rossi D, Mahmood K, Gozzini D, Mannucci B, Corana F, et al. Towards elucidating Eremurus root remedy: Chemical profiling and preliminary biological investigations of Eremurus persicus and Eremurus spectabilis root ethanolic extracts. J Med Plant Res 2015; 8(41): 1038-48. [CrossRef]
8. Gaggeri R, Rossi D, Azzolina O, Ahmed KM, Avanzini A, Mantelli M, et al. Anti-inflammatory properties of ethanolic root extract of Eremurus persicus (Jaub and Spach) Boiss, a Kurdish herbal remedy. J Med Plant Res 2013; 7(23): 1730-5.
9. Dashti M, Tavakoli H, Zarif Ketabi H, Paryab A. Ecological requirements of plant of Eremurus (Eremurus spectabilis MB) in Khorasan province. Iran J Range Desert Res 2005; 12(2): 153-65.
10. Prakash V, Sagar A. Analysis of Achyranthes aspera L. extracts for their α-amylase and urease inhibitory activities. J Drug Deliv Ther 2021; 11(2): 108-12. [CrossRef]
11. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: Properties, sources, targets, and their implication in various diseases. Indian J Clin Biochem 2015; 30(1): 11-26. [CrossRef]
12. Ganai SA. Plant-derived flavone apigenin: The small-molecule with promising activity against therapeutically resistant prostate cancer. Biomed Pharmacother 2017; 85: 47-56. [CrossRef]
13. Ganai SA, Sheikh FA, Baba ZA. Plant flavone Chrysin as an emerging histone deacetylase inhibitor for prosperous epigenetic-based anticancer therapy. Phytother Res 2021; 35(2): 823-34. [CrossRef]
14. Bortolotti M, Polito L, Battelli MG, Bolognesi A. Xanthine oxidoreductase: One enzyme for multiple physiological tasks. Redox Biol 2021; 41: 101882. [CrossRef]
15. Battelli MG, Bortolotti M, Polito L, Bolognesi A. Metabolic syndrome and cancer risk: The role of xanthine oxidoreductase. Redox Biol 2019; 21: 101070. [CrossRef]
16. Khan MA, Aslam M, Shahzad SA, Khan ZA, Khan NA, Ali M, et al. Design and synthesis of thiobarbituric acid analogues as potent urease inhibitors. J Mol Struct 2021; 1231: 129959. [CrossRef]
17. Phull AR, Ali A, Rafiq M, Tahir T, Majid A, Seo SY, et al. Antioxidant potential, urease and acetylcholine esterase inhibitory activity and phytochemical analysis of selected medicinal plants from the Republic of Korea. Explor Res Hypothesis Med 2021; 6(2): 51-59. [CrossRef]
18. Abdullahi A, Hamzah RU, Jigam AA, Yahya A, Kabiru AY, Muhammad H, et al. Inhibitory activity of xanthine oxidase by fractions Crateva adansonii. J Acute Dis 2012; 1(2): 126-9. [CrossRef]
19. Van Slyke DD, Archibald RM. Manometrie, titrimetric and colorimetric methods for measurements of urease activity. J Biol Chem 1944; 154: 623-42. [CrossRef]
20. Ganai SA, Sheikh FA, Baba ZA, Mir MA, Mantoo MA, Yatoo MA. Anticancer activity of the plant flavonoid luteolin against preclinical models of various cancers and insights on different signalling mechanisms modulated. Phytother Res 2021 Feb 13. [Epub ahead of print]. [CrossRef]
21. Karaman K, Polat B, Ozturk I, Sagdic O, Ozdemir C. Volatile compounds and bioactivity of Eremurus spectabilis (Ciris), a Turkish wild edible vegetable. J Med Food 2011; 14(10): 1238-43. [CrossRef]
22. Ghasemi S, Xu S, Nabavi SM, Amirkhani MA, Sureda A, Tejada S, et al. Epigenetic targeting of cancer stem cells by polyphenols (cancer stem cells targeting). Phytother Res 2021 Feb 22. [Epub ahead of print]. [CrossRef]
23. Karaoglan ES, Gundogdu G, Secme M, Onur S, Miloglu FD, Dodurga Y, et al. Eremurus spectabilis, rich source of isoorientin: Isolation, quantification and anti-cancer activity on SH-SY5Y neuroblastoma cells. Curr Pharm Anal 2018; 14(6): 578-85. [CrossRef]
24. Abubaker SR, Hidayat HJ. Anti tumor potential of local aslerk (Eremurus spectabilis) leaf extracts by HPLC and applying on cancer cell lines in vitro. Iraqi J Cancer Med Genet 2015; 8(2): 123-8.
25. Burmaoglu S, Ozcan S, Balcioglu S, Gencel M, Noma SAA, Essiz S, et al. Synthesis, biological evaluation and molecular docking studies of bis-chalcone derivatives as xanthine oxidase inhibitors and anticancer agents. Bioorg Chem 2019; 91: 103149. [CrossRef]
26. Xu H, Li C, Mozziconacci O, Zhu R, Xu Y, Tang Y, et al. Xanthine oxidase-mediated oxidative stress promotes cancer cell-specific apoptosis. Free Radic Biol Med 2019; 139: 70-9. [CrossRef]
27. Bircan B, Kırbağ S. Determination of antioxidant and antimicrobial properties of Eremurus spectabilis Bieb. Artvin Çoruh Üniv Orman Fak Derg 2015; 16(2): 176-86. [CrossRef]
28. Symonowicz M, Kolanek M. Flavonoids and their properties to form chelate complexes. Biotechnol Food Sci 2012; 76(1): 35-41.
29. El Aanachi S, Gali L, Rammali S, Bensouici C, Aassila H, Dari K. In vitro study of the antioxidant, photoprotective, anti-tyrosinase, and anti-urease effects of methanolic extracts from leaves of six Moroccan Lamiaceae. J Food Meas Charact 2021; 15(2): 1785-95. [CrossRef]
30. Karakaya L, Akgül Y, Nalbantsoy A. Chemical constituents and in vitro biological activities of Eremurus spectabilis leaves. Nat Prod Res 2017; 31(15): 1786-91. [CrossRef]
31. Mushtaq A, Hussein Masoodi M, Farooq Wali A, Ganai A. Total phenolic content, total flavonoid content, in vitro antioxidant activity and antimicrobial activity against human pathogenic bacteria of Eremurus himalaicus-An edible herb of North Western Himalayas. Free Radic Antiox 2017; 7(1): 90-4. [CrossRef]
32. Nile S, Keum YS, Nile AS, Jalde SS, Patel RV. Antioxidant, anti-inflammatory, and enzyme inhibitory activity of natural plant flavonoids and their synthesized derivatives. J Biochem Mol Toxicol 2017; 32(1): e22002. [CrossRef]

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Hücre Mikroenkapsülasyonunda Manuel ve Kapsülasyon Sisteminin Hücre İzolasyon Tipi ve Aljinat Yüzdesine Bağlı Verimliliğinin Karşılaştırılması

A Comparison of Cell Micro-encapsulation Efficiency of a Manually Produced and Capsulation System by Depending on the Cell Isolation Type and the Alginate Percentage

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ÖZ

Amaç: Birçok endüstriyel sektörde kullanılan polimer malzemeler sağlık bilimlerinde de farklı işlemlerde kullanılmaktadır. Bu işlemlerden biri olan enkapsülasyon sistemi hücre nakli gibi terapötik uygulamalarda tercih edilmektedir. Enkapsülasyon çalışmalarında uygulanacak yaklaşıma göre kapsül yapısında kullanılacak polimer malzeme ve oluşan kapsül boyutu değişmektedir. Aljinat, kahverengi alglerden elde edilen, içeriğindeki farklı polimerik blok oranlarına bağlı olarak değişiklik gösteren doğal polimerlerden biridir. Bu çalışmada, kapsülasyon aşaması için kullanılacak olan değişik aljinat yüzdeleri uygulanarak paratiroid hücrelerinde ideal mikroenkapsülasyon prosedürlerinin belirlenmesi amaçlanmaktadır.

Gereç ve Yöntem: Çalışmada sekonder hiperparatiroidi hastasından alınan bir adet paratiroid hiperplazi dokusundan mekanik ve enzimatik izolasyon yöntemleriyle hücre eldesi gerçekleştirilmiştir. İki farklı aljinat yüzdesi kullanarak hem manuel olarak hem de kapsülasyon cihazında otomatize olarak iki farklı akış hızı değerlendirilmiştir. Mikroenkapsüle edilen hücreler 64-79 gün boyunca *in vitro* olarak parathormon miktarları ölçülerek takip edilmiştir.

Bulgular: Değerlendirilen aljinat yüzdelerinden %2'lik konsantrasyona sahip mikroenkapsüllerin oluşturulmasında kapsülasyon cihazında kullanılan 2 mL/dk akış hızıyla morfolojik stabilite göz-

ABSTRACT

Objective: Many industrial sectors in the health sciences are using polymeric materials for different processes. One of these processes is the encapsulation system mainly preferred in therapeutic applications including cell transplantation. Depending on the main approach, the polymer type and sphere properties differ by the encapsulation method. Alginate is one of the natural polymers that show structural changes depending on different polymer ratios. In this study, our aim is to determine the ideal micro-encapsulation procedures by evaluating different alginate concentrations for the capsulation process of parathyroid cells.

Material and Method: In this study, cell isolation of single parathyroid hyperplasia tissue from a patient with secondary hyperparathyroidism was performed by using two methods including mechanical and enzymatic isolation. Two different alginate percentages were used for micro-encapsulation which created manually, and a capsulation system made with two different flow rates. Parathormone levels of micro-encapsulated cells were followed for 64-79 days *in vitro*.

Results: Morphological stability was observed for 2% alginate concentration with a flow rate of 2 mL/min used group from the micro-encapsulation system. However, parathyroid hormone re-

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lenmiştir. Ayrıca parathormon salınımı açısından hücre izolasyon tipi ve aljinat yüzdeleri arasında benzer sonuçlar elde edilmiştir.

Sonuç: Uzun süreli mikroenkapsülasyon verimliliğinin arttırılması için yapısal ve fonksiyonel açıdan birçok parametrenin belirlenmesi gerekmektedir. Bu çalışma ile enzimatik izolasyon metoduyla elde edilen paratiroid hücrelerinin kapsülasyon sistemi kullanılarak artan akış hızında daha stabil bir yapı oluşturdukları belirlenmiştir.

Anahtar Kelimeler: Paratiroid, mikroenkapsülasyon, ultra saf aljinat, kapsülasyon sistemi, hücre izolasyonu.

lease showed similar results between cell isolation type and alginate percentages.

Conclusion: Increasing the long-term micro-encapsulation efficiency, certain criteria should be determined from structural and functional aspects. In this study, the micro-encapsulation of parathyroid cells obtained by the enzymatic isolation method formed a more stable structure at increasing flow rate by using the capsulation system.

Keywords: Parathyroid, micro-encapsulation, ultra-pure alginate, capsulation system, cell isolation

GİRİŞ

Polimer malzemeler günümüzde birçok endüstriyel sektörde yapıştırıcı olarak ayrıca paketleme, boyama ve kaplama gibi farklı amaçlarla da kullanılmaktadır (1). Bu kullanım alanlarının yanı sıra, polimer malzemeler farmasötik, gıda, kozmetik ve sağlık bilimleri alanlarında enkapsülasyon gibi biyomühendislik uygulamalarında kullanılmaktadır (2). Enkapsülasyon işlemi için kullanılacak olan polimer malzemeler doğal polimerler ve sentetik polimerler olarak iki grupta incelenmektedir. Sentetik polimerlere polietilen glikol, polivinil alkol, poliüretan, polietersülfon, polipropilen, sodyum aljinat gibi birçok örnek verilebilir. Doğal polimerlerin yapımında kullanılan doğal kaynaklara selüloz, kolajen, kitosan ve aljinat gibi malzemeler örnek olarak gösterilebilir (3). Polimer malzemeler kullanılarak gerçekleştirilen enkapsülasyonun ana hedefleri enkapsüle edilen biyolojik maddenin dış çevreden korunmasını sağlamak ve mekanik destek ile yapısal stabilite yaratmaktır (4).

Biyouyumluluk, kullanılacak olan malzemenin biyolojik objelere ve biyolojik objenin kullanılan malzemeye karşı oluşturacağı tolerans olarak tanımlanmaktadır (5). Hücresel terapi veya ilaç taşınımı gibi çalışmalarda kullanılacak enkapsüle edilmiş terapötik ajanların işlevini yerine getirebilmesi ve tedavinin uzun süreli olabilmesi için kapsülasyon aşamasında kullanılan polimer malzemenin biyouyumluluk özelliği önem kazanmaktadır. Biyouyumluluğu ile ön plana çıkmış olan ve FDA tarafından da onaylı aljinat polimeri, enkapsülasyon çalışmalarında en sık kullanılan biyomateryaldir (3).

Aljinat, kahverengi alglerden elde edilen anyonik bir polisakkarittir. Yapısında β-D-mannuronik (M) ve α-L-guluronik (G) asit blokları içermektedir (6). Yapısında fazla miktarlarda guluronik asit blokları içeren ve aljinatın daha saf hali olan ultra saf aljinat, yapısal olarak daha sert, stabil ve biyouyumluluğu daha yüksek olmaktadır (2). Ultra saf aljinat bu özellikleri sayesinde tip I diyabet (7,8), deneysel olarak tiroit dokusunun kapsüle edilmesi (9), kalıcı hipoparatiroidi tedavisi için paratiroid hücrelerinin kapsülasyonu (10, 11) gibi birçok enkapsülasyon temelli çalışmalarda tercih edilmiştir.

Kalıcı hipoparatiroidi (KH), iatrojenik nedenlerden dolayı paratiroid dokularının kanlanmasının bozulması yahut paratiroidin tamamen alınması nedeniyle ortaya çıkan bir hastalıktır. Türkiye'de kalıcı hipoparatiroidi hastalığının görülme sıklığı, endemik guatr ülkesi olması nedeniyle daha fazladır (12). KH hastalığının neticesinde, hipokalsemi, hiperfosfatemi ve düşük parathormon (PTH) seviyesi gibi ciddi klinik semptomlar gelişmektedir (13). Hastaların semptomatik tedavi olarak hayatları boyunca ilaç kullanması gerekmektedir. İlaç tedavisi kesin çözüm olmamakla birlikte birçok yan etkiyi de içinde barındırmaktadır. KH hastalığı için semptomatik tedavi haricinde paratiroid nakli küratif olarak uygulanabilen tek tedavi seçeneğidir. Tarihsel seyir açısından incelenecek olursa, 1911 yılında ilk paratiroid nakli gerçekleştirilmiştir (14). Bu nakilden sonra birçok farklı gereç ve yöntem kullanılarak nakil işlemleri yapılmıştır. Naklin sağkalım süresini uzatmak amacıyla yapılan ilk kapsüle edilmiş paratiroid hücre nakli 1997 yılında gerçekleştirilmiştir (15). Paratiroid nakli, günümüzde de halen uygulanmakta ve geliştirilmesi için birçok çalışma yapılmaktadır (3).

Bu çalışmada, paratiroid hücrelerinin mikroenkapsülasyon aşamasında kullanılacak olan ideal ultra saf aljinat yüzdesinin belirlenmesi, hücre izolasyon metodunun buna bağlı verimliliği ve manuel ve kapsülasyon cihazı kullanılarak oluşturulan kapsüllerin verimliliğinin karşılaştırılarak değerlendirilmesi amaçlanmıştır.

GEREÇ VE YÖNTEM

Çalışma, Bezmialem Vakıf Üniversitesi girişimsel olmayan araştırmalar etik kurulu onayı ile Helsinki Deklarasyonu prensiplerine uygun olarak gerçekleştirilmiştir (Kabul numarası: 54022451-050.05.04-522). Çalışma kapsamında kullanılan paratiroid dokusu için hastadan imzalanmış gönüllü onam formu alınarak deneysel süreçler başlatılmıştır.

Hücre İzolasyonu

Çalışma sürecinde sekonder hiperparatiroidi hastasından alınmış tek bir paratiroid dokusu kullanıldı. Doku eşit olacak şekilde ikiye kesilerek hem mekanik yolla hem de enzimatik yolla hücre izole edildi. Hücre izolasyonlarını gerçekleştirmek için Yucesan ve ark. uyguladığı prosedürler takip edildi (2,16). Muse hücre analiz cihazı (Merck Millipore, Germany) kullanılarak hücrelerin canlılık tayinleri belirlendi.

Mikroenkapsülasyon

Mikroenkapsülasyon aşamalarında kullanılacak olan ultra saf aljinat maddesi (Pronova UP MVG; Nova Matrix, Oslo, Norway) %1,5 ve %2 oranlarında hazırlandı. Her iki oranda hazırlanan aljinat maddesi enzimatik ve mekanik olarak iki farklı izolasyon yöntemiyle elde edilen hücreler ile önceden yapılmış çalışmalarla belirlenen %28 (hücre/hücre+aljinat) oranında her grupta $10x10^6$ hücre olacak şekilde ayrı ayrı karıştırıldı. Çalışma kapsamında mikroenkapsülasyon işlemi manuel bir şekilde ve kapsülasyon cihazı kullanılarak uygulanmıştır.

İlk olarak, hazırlanan hücre-aljinat karışımı kullanılarak manuel enkapsülasyon işlemi gerçekleştirildi. Bu aşamada, karışım 300 mOsm $CaCl_2$ içeren çözeltiye küt uçlu 15 *gauge* şırınga ucu kullanılarak el ile yavaşça damlatıldı. Çözelti içerisindeki oluşan kapsüller izotonik su ile yıkanarak ortamdaki fazla kalsiyum uzaklaştırıldı. Kapsüller McCoy's (Gibco, Thermo, MA, USA) hücre kültür medyumu içerisine aktarılarak nemli inkübatörde 37°C'de ve %5 CO_2'de 64 gün boyunca kültüre edildi.

İkinci olarak, kapsülasyon cihazı (Pump 11 Pico Plus Elite Infusion/Withdrawal Programmable Dual Syringe Pump, Harvard Apparatus™, Holliston, MA, USA) kullanılarak hücre-aljinat karışımı 300 mOsm $CaCl_2$ içeren çözeltiye 1,5 mL/dk ve 2 mL/dk hızlarında damlatıldı. Damlatma işlemi küt uçlu 25 *gauge* şırınga ucu kullanılarak gerçekleştirildi. Kapsüller izotonik su ile yıkanıp McCoy's (Gibco, Thermo, MA, USA) hücre kültür medyumuna alındı. Nemli inkübatörde 37°C'de ve %5 CO_2'de kültüre edilen kapsüller 79 gün boyunca takip edildi.

PTH Seviyesinin Belirlenmesi

Çalışma süresince manuel olarak, 1,2 mL/dk ve 1,5 mL/dk cihaz hızlarıyla oluşturulan %1,5'lik aljinatın kullanıldığı kapsüller 1, 7, 15, 21, 28, 35, 42, 49, 57 ve 64. günlerde; %2'lik aljinatın kullanıldığı kapsüller ise 1, 7, 15, 21, 28, 35, 42, 50, 57, 64, 72 ve 79. günlerde süpernatant örnekleri toplandı. Değerlendirilen takip süreci, PTH salınımının sonlandığı noktaya kadar gerçekleştirilmiştir. Toplanan süpernatantlardan PTH miktarları insan PTH ELISA kiti (RayBiotech Inc., Norcoss, GA, USA) kullanılarak belirlendi. Absorbans ölçümleri 450 nm'de Mark Microplate Absorbance Reader (Bio-Rad, USA) cihazı kullanılarak gerçekleştirildi.

İstatistiksel Değerlendirme

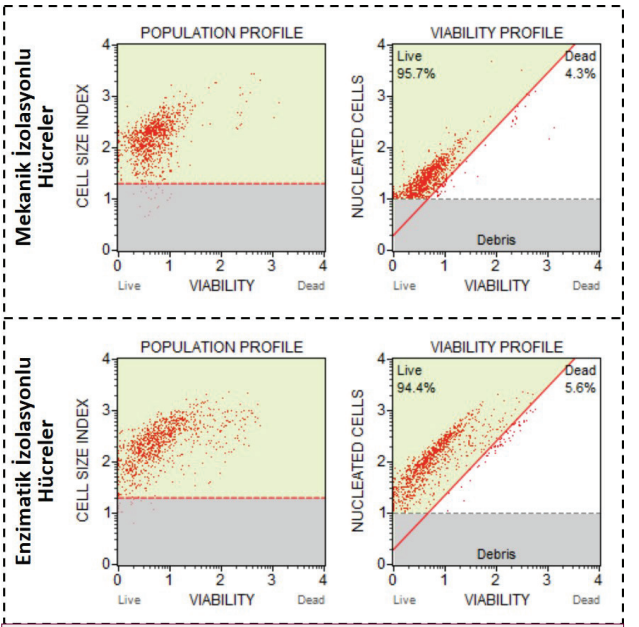
Çalışma kapsamında belirlenen PTH değerleri hem zaman hem aljinat yüzdesi ek olarak aljinat yüzdesi ve uygulanan teknikler açısından karşılaştırılması, çift yönlü ANOVA testi (*Tukey's multiple comparisons test* ve *Sidak's multiple comparisons test*) ile gerçekleştirilmiştir. $p<0,05$ olduğu durum anlamlılık olarak değerlendirilmiştir.

BULGULAR

Çalışmada sekonder hiperparatiroidi hastasından alınan bir paratiroid hiperplazi dokusu kullanıldı. Mekanik ve enzimatik izolasyon yöntemleriyle hücre izolasyonunu gerçekleştirmek için doku iki eşit parçaya bölündü. Akış sitometrisinde mekanik izolasyon ile elde edilen hücrelerin %95,7 canlılığa ve 67,6x10^6 hücre sayısına sahip olduğu belirlendi (Şekil 1). Devamında, enzimatik izolasyon ile elde edilen hücreler incelendiğinde %94,4 canlılığa ve toplam 70,2x10^6 hücre sayısına sahip oldukları tespit edildi.

Morfolojik Görünümleri

Kapsülasyon, manuel olarak ve kapsülasyon cihazında 1,2 mL/dk ve 2 mL/dk hızları ile gerçekleştirildi. Ardından, izotonik su çözeltisi ile yıkanan kapsüller kültüre edildi. %1,5'lik aljinat kullanılan gruplar 64 gün, %2'lik aljinat kullanılan gruplar 79 gün boyunca kültür ortamında takip edildi. Oluşturulan kapsüller, kültüre edilmeden önce ışık mikroskobu ile gözlemlendi (Şekil 2). İlk güne ait görüntülerde, %1,5'lik aljinat gruplarında manuel bir şekilde oluşturulan kapsüllerin çapları >2mm olduğu belirlendi. Aynı durum kapsülasyon cihazında akış hızı 1,2 mL/dk kullanılarak oluşturulan kapsül örneklerinde izolasyon tipi fark etmeksizin birbirine yakın boyutlarda oluştuğu görüldü. Kapsülasyon cihazında akış hızı 2 mL/dk olan grupta ise kapsül boyutlarının küçüldüğü gözlemlendi. %2'lik aljinat ile hazırlanan gruplarda da benzer sonuçlar elde edildi. Manuel olarak ve kapsülasyon cihazında akış hızı 1,2 mL/dk olan kapsül örneklerinde kapsüllerin boyutlarının benzer olduğu, akış hızı 2 mL/dk olan kapsül boyutlarının hızın artışına bağlı olarak küçüldüğü tespit edildi. %2'lik aljinat grubundaki enzimatik izolasyonlu örneklerde hücre yoğunluğunun daha az olduğu kapsülün şeffaf bir görünüme sahip olmasından anlaşılmaktadır (Şekil 2).

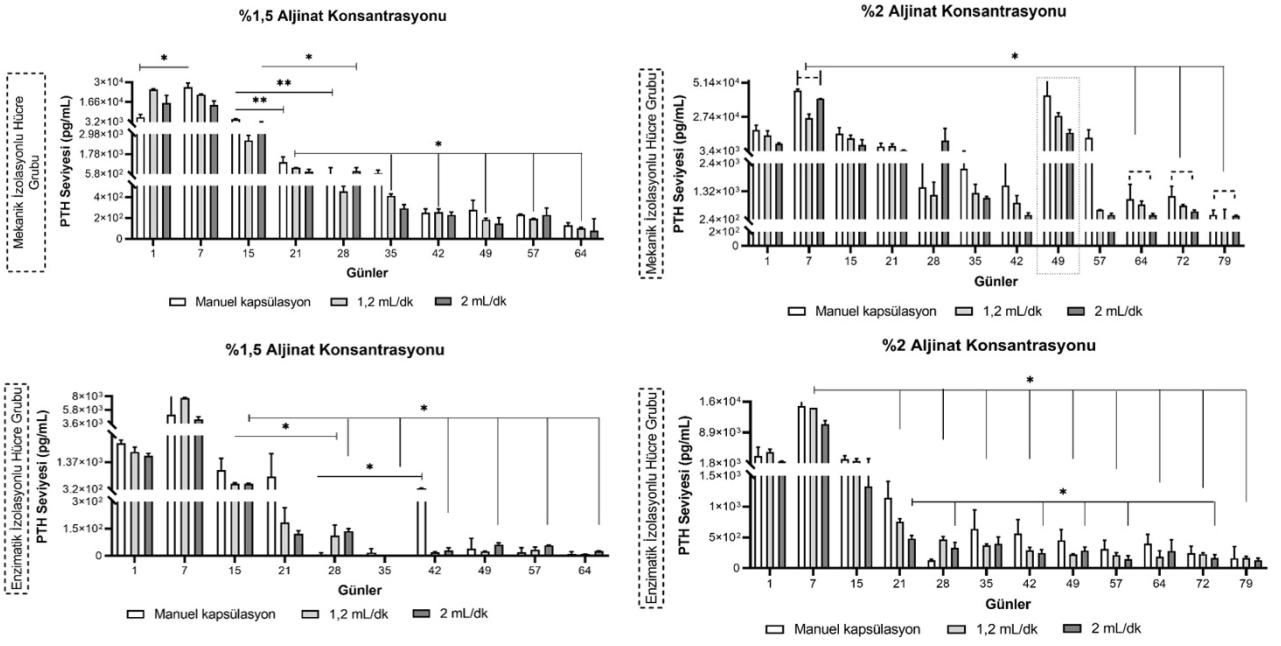


Şekil 1. Paratiroid dokusundan mekanik ve enzimatik olarak iki farklı izolasyon tipi kullanılarak izole edilen hücrelere ait popülasyon ve canlılık profilleri

Takip süreçlerinde; %1,5 aljinat konsantrasyonu uygulanan enzimatik izolasyonlu hücrelerin kapsüle edildiği grupta kültürün 28. gününden itibaren yüzeysel çatlamalar oluşmuştur. Bu çatlamaların derinlikleri manuel olarak oluşturulan grupta daha fazladır. %2'lik aljinatın değerlendirildiği grupta ise; mekanik izolasyonlu hücre gruplarında 49. günden sonra çatlayan yüzeysel alanların olduğu gözlenmiş fakat enzimatik izolasyonlu hücre gruplarında herhangi bir yapısal bozulma belirlenmemiştir.

PTH Salınımına %1,5 Aljinat Konsantrasyonunun Etkisi

Mikroenkapsüllerin fonksiyonel olup olmadıkları salınan PTH miktarı ile belirlenmiştir. Mekanik izolasyonlu hücrelerin kullanıldığı; ilk grupta manuel yöntemle yapılan kapsüllerin PTH seviyesi 1. ve 7. günde anlamlı şekilde artmıştır ($p=0,0443$). 15. günden itibaren, 21. ve 28. güne kadar devam eden süreçte PTH seviyesi anlamlı bir şekilde azalmaya başlamıştır ($p=0,0211$). Bu azalan PTH seviyesi aynı şekilde akış hızı 1,2 mL/



Şekil 2. El ile manuel olarak ve kapsülasyon cihazında iki farklı akış hızı (1,2 mL/dk ve 2 mL/dk) oluşturulan mikroenkapsüllerin ışık mikroskobu görüntüleri (büyütme oranı 4X). Farklı aljinat oranlarının kullanıldığı gruplarda hem mekanik hem de enzimatik izolasyon ile elde edilen hücreler kullanılmıştır. Üst panelde kapsülasyon işleminde %1,2'lik aljinatın kullanıldığı gruplara ait kapsüller gösterilmektedir. El ile manuel olarak ve kapsülasyon cihazının 1,2 mL/dk akış hızında oluşturulan kapsüllerin boyutları arasında bir fark görülmemektedir. Cihaz akış hızı 2 mL/dk olarak uygulandığında kapsül boyutlarının küçüldüğü gözlemlenmektedir. Alt panelde kapsülasyon işleminde %2'lik aljinatın kullanıldığı gruplara ait kapsüller gösterilmektedir. Bu grupta, %1,2'lik aljinatın kullanıldığı grupta olduğu gibi benzer kapsül formasyonu gözlemlenmiştir. %2'lik aljinatın kullanıldığı enzimatik izolasyonlu hücrelerin kapsüle edildiği 2 akış hızına ait grupta kapsüller beklenenden küçük olduğu için 0,2µm çapındaki hücre *strainer* kullanılarak kültür takibi yapılmıştır.

dk olan grupta da 21. günden itibaren başlayarak aynı azalma örüntüsünü göstermiştir (p=0,0211-35.gün; p=0,0382-49.gün; p=0,0211-57.gün; p=0,0214-64.gün). Bu değişim akış hızı 2 mL/dk olan grubu için azalarak devam eden bir süreç gösterse de yalnızca 15. günden 28. güne kadar olan süre için PTH seviyesi azalmıştır (p=0,0201) (Şekil 3).

İkinci grup olarak enzimatik izolasyonlu hücrelerin kullanıldığı kapsüllerden elde edilen PTH seviyesi; manuel olarak oluşturulan kapsüller için azalan bir parametre gösterse de 28. gün ve 42. gün aralığında artan PTH salınımı gözlenmiş ve istatistiksel olarak anlamlı bulunmuştur (p=0,0217). Süregelen takip boyunca, 42. gündeki artış 64. güne kadar yapılan zaman diliminde tekrar gözlenmemiştir.

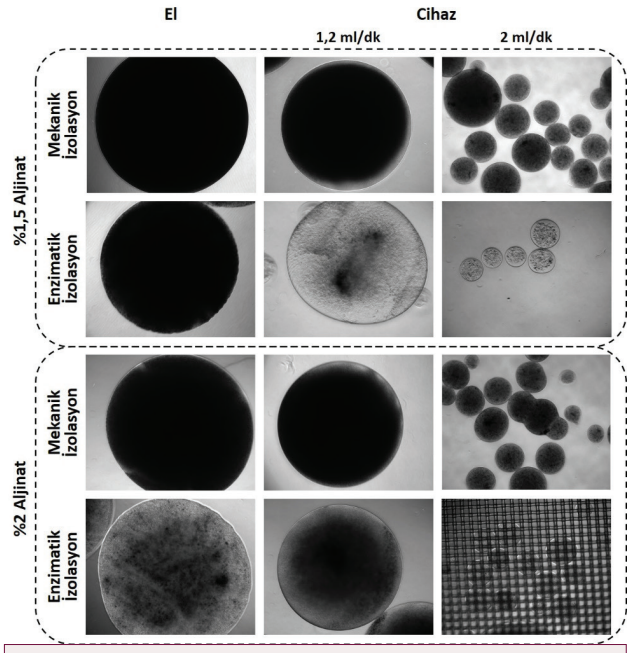
Cihaz ile oluşturulan kapsüllerden akış hızı 1,2 mL/dk olan grupta 15 ve 28. günler aralığında anlamlı bir şekilde PTH seviyesinde azalma gözlenmiştir (p=0,0331). Buna bağlı olarak 64 güne kadar düşmeye devam etmiştir. Morfolojik olarak bu gruplarda kapsüllerde bir çatlama belirlenmemiştir. Akış hızının 2 mL/dk olarak değerlendirildiği kapsüllerin 15. günden itibaren PTH salınımı anlamlı şekilde azalmaya devam ettiği fakat morfolojik olarak stabil bir yapı gösterdikleri belirlenmiştir (p=0,0211-21. gün; p=0,0373-28. gün; p=0,0211-35 ve 49. günler; p=0,0347-57. gün; p=0,0329-64. gün) (Şekil 3).

PTH Salınımına %2 Aljinat Konsantrasyonunun Etkisi

Salınan PTH miktarı açısından; %2'lik aljinat konsantrasyonu hücre izolasyon tipine göre farklılıklar göstermiştir.

Mekanik izolasyonlu hücreler manuel olarak veya kapsülasyon cihazı kullanılmasından bağımsız bir şekilde kültürün 7. gününden sonra azalan bir eğri göstermiştir. Bu azalan parametre istatistiksel olarak; manuel yolla üretilen ve akış hızı 2 mL/dk olan grupta kültürün 64., 72. ve 79. günlerinde anlamlılık gözlenmiştir (manuel olarak oluşturulan grup için p=0,0272-64. gün; p=0,0225-72. gün; p=0,0211-79. gün ve akış hızı 2mL/dk olan grupta ise p=0,0211-64., 72. ve 79. günler) (Şekil 3). İstisnai olarak 49. günde değerlendirilen üç metot için artan PTH seviyesi belirlenmiş fakat istatistiksel olarak anlamlı bulunmamıştır (p>0,05).

Enzimatik yöntemle izole edilen hücreler; manuel olarak kapsülasyon uygulandığında takip süresince azalan bir PTH salınımı göstermiş fakat istatistiksel olarak anlamlı bulunmamıştır (p>0,05). Kapsülasyon cihazının değerlendirildiği grupların ilki olan akış hızı 1,2 mL/dk olan grupta ise 7. günden 79. güne kadar olan süre de anlamlı şekilde PTH salınımı azalmıştır (p=0,0211/tüm zamanlar). Akış hızı 2ml/dk olan grupta ise 21. günden sonra azalan parametrede anlamlılık belirlenmiştir (p=0,0211-28 ve 42. gün; p=0,0215-49. gün; p=0,0211-57 ve 72. gün) (Şekil 3).



Şekil 3. İki farklı aljinat konsantrasyonu ve iki farklı izolasyon metodu ile elde edilen hücrelerin kullanıldığı mikroenkapsüllerin PTH salınım seviyelerinin takibi. Üst sol panelde %1,5 aljinat kullanılarak mekanik izolasyona tabi tutulmuş hücrelerin kapsülasyonu gösterilmiştir. Alt sol panelde %1,5 aljinat kullanılarak enzimatik izolasyona tabi tutulmuş hücrelerin kapsülasyonu gösterilmiştir. Üst sağ panelde %2 aljinat kullanılarak mekanik izolasyona tabi tutulmuş hücrelerin kapsülasyonu gösterilmiştir. Alt sağ panelde %2 aljinat kullanılarak enzimatik izolasyona tabi tutulmuş hücrelerin kapsülasyonu gösterilmiştir. PTH: Parathormon, pg/mL: pikogram/mililitre. *p<0,05. **p<0,001.

Kültür ortamındaki takip süresince, toplanan süpernatant örneklerindeki PTH miktarlarına ait detaylar Tablo 1'de gösterilmiştir.

TARTIŞMA

Aljinat, çok sayıda alg türünden izole edilebilir ve ana yapıları, dalsız ikili bloktan oluşan kopolimerler olarak tanımlanır. Bu blokları oluşturan yapılar; mannuronik ve guluronik asit bloklarıdır (3). Divalent katyonlar sayesinde farklı bağlanma sağlayan bu bloklar farklı por özelliklerine sahip küre şeklinde yapılar oluşturabilirler. Özellikle bu çalışma kapsamında kullanılan ultra saf aljinat %60'tan fazla guluronik asit bloğu içeriğine sahiptir. Aljinatın bağ yapılarını oluşturma yetilerine göre divalent katyonlar sırasıyla $Pb^{2+}>Cu^{2+}>Cd^{2+}>Ba^{2+}>Sr^{2+}>Ca^{2+}>Co^{2+}>Ni^{2+}>Zn^{2+}>Mn^{2+}$'dır (17-19). Bu divalent katyonlar kullanıldıklarında ultra saf aljinatın yüksek guluronik asit içeriği sayesinde yumurta kutusu benzeri (*egg-box like*) por yapıları sağlayan küre formunu oluşturmaktadır (3,17).

Bugüne kadar paratiroid dokusunun/hücrelerinin kapsülasyonu *in vitro* ve *in vivo* birçok çalışmada değerlendirilmiştir (2,10,15,20-25). Paratiroid hücrelerine spesifik çalışmalarıyla Hasse ve ark. baryum aljinat, mitojenik aljinat ve hem *in vitro* hem de *in vivo* için amitojenik aljinat dahil olmak üzere farklı aljinat türevlerini karşılaştırmıştır. Bu konudaki çalışmaların çoğunda ksenotransplantasyon uygulanmış ve 30 haftaya kadar takip ederek sonuçları literatüre katmıştır (10,15,20,21,23,24).

Bunlardan ilki; Hasse ve ark. 1994 yılında immün sistemden kaçmak için mikroenkapsüle ettikleri paratiroid dokularını hipokalsemik sıçanlara naklettikleri çalışmadır. Sıçanların 90 günlük takibi boyunca normal değerlerde serum kalsiyum seviyesi gözlemlenmiştir. Bu bilgiler ışığında, mikroenkapsüle naklin immün yanıttan kaçarak uzun süreli sağkalımı sağladığı sonucuna ulaşılmıştır (20). Güncel olarak son yıllarda yapılan iki *in vitro* çalışma daha bulunmaktadır; Yucesan ve ark. 2017 yılında yayınladıkları bir çalışmada kapsülasyon için kullanılacak farklı paratiroid hücrelerinin sayısını değerlendirmişlerdir. 75 günlük *in vitro* takip sonucunda $20x10^6$ paratiroid hücresinin aljinat ile kapsülasyonunun daha kararlı bir morfoloji gösterdiğini bildirmişlerdir (2).

Ayrıca, kapsüle edilmiş paratiroid dokusu/hücresi klinik uygulamalarda da kullanılmıştır. Kapsüle edilmiş paratiroid nakli, günümüze kadar on iki alıcı için uygulanmış ve yalnızca yedi vaka raporunda bildirilmiştir. İlk olarak, 1997 yılında Hasse ve ark. iki alıcı için ilk mikroenkapsüle paratiroid naklini gerçekleştirmiş ve üç aylık nakil sağkalımı bildirmiştir (22). İkinci nakil, Zimmerman ve ark.'nın 2001 yılında bir alıcı için gerçekleştirdikleri nakildir. Sağkalım açısından naklin üçüncü ayından sonra, alıcıda implantasyon alanından histolojik numune alınmış ve ne paratiroid doku partikülleri ne de mikrokapsüllere ait bir iz gözlemlemediklerini bildirilmiştir (26). Üçüncü çalışma, yine 2001 yılında Tibell ve ark.'nın uyguladıkları makrokapsül ile paratiroid doku partiküllerinin dört alıcıya naklidir ve bir yıla kadar nakil sağkalımı bildirilmiştir (27). Dördüncü nakil sunumunda, Ulrich ve ark. iki alıcının PTH seviyelerinde yükselmeler olduğunu ve daha sonra iki alıcının günlük ilaç gereksinimini yarı doza düşürdüğünü bildirmiştir (28). 2009'da Cabane ve ark. bir alıcıda enzimatik olarak izole edilmiş paratiroid hücrelerini mikroenkapsüle ederek nakil gerçekleştirdiler ve 20 aylık nakil sağkalımı ile en uzun takip verilerini bildirmiştir (29). Daha sonra Khryshchanovich ve ark. makroenkapsüllenmiş paratiroid doku parçalarını nakletmiş ve üç aylık takip verilerini literatüre sunmuştur (30). Mikroenkapsüle paratiroid hücre nakli ile ilgili son ve yedinci vaka bildirisi Yucesan ve ark. tarafından 2019 yılında bir alıcı için gerçekleştirilmiş ve nakil sonucunda bir yıl boyunca takip edilmiştir ve hasta günlük kullandığı ilaçların tamamını bıraktığı bildirilmiştir (11).

Bu çalışmada elde edilen verilere bakıldığında; manuel kapsülasyon açısından aljinat yüzdesi fark etmeksizin mekanik izolasyon grupları, takip süresinde 15. günden itibaren PTH salınımının anlamlı şekilde değiştiği belirlendi. Bunun en önemli nedenlerinden birisi olarak kapsül alanı içerisindeki hücrelerin ve/veya doku parçalarının yeterince besine erişim sağlayamamaları ihtimal dahilindedir. Ayrıca belirlenen günlerde azalan örüntüye rağmen yeniden bir PTH artışı gözlenmiştir. Bu artış morfolojik

Tablo 1. Mekanik ve enzimatik olarak elde edilen paratiroid hücreleri %1,5 ve %2'lik aljinat birlikte el ile manuel olarak ve kapsülasyon cihazında iki farklı akış hızında (1,2 mL/dk ve 2 mL/dk) oluşturulan mikroenkapsüllerin kültür boyunca belirli günlerde salgıladıkları PTH miktarlarının sonuçları. PTH: Parathormon, pg/mL: pikogram/mililitre, mL/dk: mililitre/dakika.

%1,5'lik Aljinat			**1. Gün**	**7. Gün**	**15. Gün**	**21. Gün**	**28. Gün**	**35. Gün**	**42. Gün**	**49. Gün**	**57. Gün**	**64. Gün**
Mekanik İzolasyonlu Hücreler	**El ile Manuel**		7716	28686	4674	1064	284	500	227	215	237	111,5
			5060	24806	5246	1522	848	756	278	344	228	148,5
	Kapsülasyon Cihazı	**1,2 mL/dk**	24738	21912	2826	972	490	426	232	173	192	99
			25478	21398	2390	950	420	400	278	195	196	109,5
		2 mL/dk	12360	12728	3196	818	896	318	250	188	278	161
			19628	16514	2846	618	586	268	210	106	186	0
Enzimatik İzolasyonlu Hücreler	**El ile Manuel**		2010	7634	738	187,6	14,8	2,1	359	80	37,4	19,2
			2186	2464	1384	1438	2,2	33,7	333	0	0	0
	Kapsülasyon Cihazı	**1,2 mL/dk**	1648	7694	558	242,3	153,3	2,3	21,2	26,2	23,2	9,5
			1896	7836	500	126,9	73,1	1,2	16,4	22,3	44,6	8,2
		2 mL/dk	1538	4574	520	109	146,6	1,7	21,2	53,5	55,4	24,3
			1678	3982	550	134,2	126,3	1,8	40,2	68,9	59,4	27,2

%2'lik Aljinat			**1. Gün**	**7. Gün**	**15. Gün**	**21. Gün**	**28. Gün**	**35. Gün**	**42. Gün**	**49. Gün**	**57. Gün**	**64. Gün**	**72. Gün**	**79. Gün**
Mekanik İzolasyonlu Hücreler	**El ile Manuel**		16005	46608	18652	5004	2226	1260	2254	27986	8730	614	873	547
			20609	45308	13010	8252	742	3132	848	57144	16684	1426	1409	299
	Kapsülasyon Cihazı	**1,2 mL/dk**	12481	24447	10616	6014	848	1028	684	26600	616	909	805	519
			16644	28510	13840	8078	1534	1500	1082	29606	608	723	737	0
		2 mL/dk	8999	40252	10378	4026	16782	1034	476	14786	384	456	589	393
			8278	40216	5276	3696	4928	1112	366	17814	465	379	526	381

%2'lik Aljinat			**1. Gün**	**7. Gün**	**15. Gün**	**21. Gün**	**28. Gün**	**35. Gün**	**42. Gün**	**49. Gün**	**57. Gün**	**64. Gün**	**72. Gün**	**79. Gün**
Enzimatik İzolasyonlu Hücreler	**El ile Manuel**		2053	11172	2294	1330	118	856,6	723,5	577,5	414,6	505,6	324,2	296,7
			4938	18883	3336	954	140	418,7	409,3	332,3	213,7	292,7	163,2	28,6
	Kapsülasyon Cihazı	**1,2 mL/dk**	4846	14573	1870	720	434	363,6	327	231,8	242,5	121,9	243,6	187,9
			3923	14574	2702	790	500	388,4	256,1	219,5	182,6	256,7	217	150,1
		2 mL/dk	2170	11424	2422	518	394	475,9	286,8	326,6	188	408,4	205,2	154,1
			2308	10188	236	448	270	318,1	211,1	249,3	113,6	153	131,8	111,2

olarak kapsüllerin çatlamaya başladığı zaman diliminde belirlenmiştir ve bu artışın kapsül alanı içerisinde henüz salınmamış PTH'nın veya kapsül alanından ayrılan serbest hücrelerin açığa çıkmasından kaynaklandığı düşünülmektedir. Benzer bir bakış açısı ile enzimatik izolasyon değerlendirildiğinde aljinat yüzdesi anlam kazanmıştır. Düşük aljinat konsantrasyonunun kullanıldığı grupta (%1,5 aljinat) azalan PTH salınımının, dalgalanan bir seyir gösterdiği ve özellikle manuel olarak üretilen kapsül gruplarında bu dalgalanma belirgin olarak görülmüştür. Morfolojik olarak yine bu grubun 28. günde ortaya çıkan yüzeysel çatlamalarının, dalgalanan seviyeyi etkilediği düşünülmektedir. Daha yüksek konsantrasyonda değerlendirilen aljinat grubunun (%2) mekanik hücrelerin kapsüle edildiği ve cihaz sistemi kullanılan gruplarda da aynı morfolojik düzensizleşmeyle beraber, belirli bir noktada artış ve yeniden azalan bir PTH salınım sonucu vermiştir. Bu ani artışın, yine kapsül yüzeyinde oluşan çatlama sebebiyle olduğu düşünülmektedir. Bilindiği üzere, mekanik izolasyonla elde edilen hücrelerin mikro-doku parçaları halinde bulundukları ve çatlama alanlarından ayrılan bu mikro-doku parçalarının süpernatant içerisindeki PTH miktarını doğrudan etkilemesi elde edilen verilerden de anlaşılmaktadır.

Kapsülasyon sistemi kullanılarak oluşturulan kapsül grubunda, aljinat yüzdesinden bağımsız olarak mekanik izolasyona tabii tutulan hücrelerin kapsüle edildiği grupta PTH salınım seviyeleri 15 ila 21. gün aralığından başlayarak değişmiştir. Enzimatik izolasyonlu hücrelerin kullanıldığı gruplarda, aljinat yüzdesi ve kapsülasyon sisteminin akış hızı morfolojik anlamda farklılık göstermiştir. %1,5 ve %2'lik aljinat grubunda 21-28 günlere ait zaman diliminde PTH salınımı her iki akış hızında azalma göstermiştir. Buna rağmen morfolojik stabilite yalnızca %2'lik aljinat ile ve akış hızının 2 mL/dk olduğu grupta korunmuştur. Ayrıca deneyin yapılan takip süreçlerine baktığımızda hücrelerin salgıladığı PTH miktarı %1,5 aljinat konsantasyonu için 64 güne kadar olup, %2'lik aljinat konsantasyonu ise 79 güne kadar gerçekleştirilebilmiştir. Hücrelerin beslenebilme süreci %2'lik aljinat konsantrasyonu için daha uzun devam edebilmiştir.

Çalışmanın bazı kısıtlılıkları da mevcuttur. Özellikle kapsüle edilmemiş hücrelere ait *in vitro* takipler PTH seviyesi açısından değerlendirilememiştir. Bunun tek nedeni, yalnızca bir paratiroid dokusundan elde edilen hücrelerle, bütün grupların belirlenen limitlerde hem kapsül hem de çıplak hücre kültürü için sayıca yeterli gelmemesidir. Ayrıca kapsül içerisindeki hücrelerin canlılık tayinleri için floresan boyalarının uygulanarak canlılık açısından gözlenememesi bir başka kısıtlılıktır. Fakat çalışma süresince hem DAPI (moleküler ağırlığı 277,324 g/mol) hem de Hoechst 33342 (moleküler ağırlığı 452,6 g/mol) floresan boyaları kullanılarak kapsül alanı içerisindeki hücrelerin canlılıkları değerlendirilmek istenmiştir. Bu durum boyaların moleküler ağırlıklarının ultra saf aljinatın por alanlarından giremeyecek kadar büyük olabileceği ihtimalini düşündürmektedir. İlerleyen çalışmalar için daha düşük moleküler ağırlıklı floresan boyalar denenmesi planlanmaktadır.

Sonuç olarak; kapsülasyon sistemlerinin uzun vadede verimliliğinin arttırılması için yapının korunması ve gereken parametrelerin optimize edilmesi sağlanmalıdır. Özellikle yüzey alanının arttırılması ve bunun uygun biyolojik olarak uyumlu polimer konsantrasyonu ile dengelenerek hücrelerin etkili beslenmesine olanak sağlanması gerekmektedir. Yapılan çalışma ile paratiroid dokusu hücreleri için uygun hücre izolasyon tipinin enzimatik hücre izolasyonu olduğu, %2'lik aljinat konsantrasyonunun daha uzun hücre sağkalımını desteklediği ve kapsülasyon sisteminin 2mL/dk akış hızı ile daha stabil bir yapı oluşturduğu belirlenmiştir. İlerleyen çalışmalar ile daha çok sayıda paratiroid hücresi kullanılarak, belirlenen kriterlerin daha uzun süreli takiplerle değerlendirilmesi ve optimum koşulların tespit edilmesi hedeflenmektedir.

Teşekkür: Ekipman desteği için Bezmialem Vakıf Üniversitesi Tıbbi Biyoloji AD. 'dan Prof. Dr. Fahri Akbaş'a teşekkür ederiz.

Hakem Değerlendirmesi: Dış bağımsız.

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KAYNAKLAR/REFERENCES

1. Candlin JP. Chapter 3 Polymeric Materials: Composition, Uses and Applications. In: Chalmers JM, Meier RJ, editors. Comprehensive Analytical Chemistry 53: Elsevier; 2008. p. 65-119. [CrossRef]
2. Yücesan E, Başoğlu H, Göncü B, Kandaş N, Ersoy Y, Akbaş, F, et al. In-vitro optimization of microencapsulated parathyroid cells. Dicle Medical Journal 2017; 44(4): 373-80. [CrossRef]
3. Goncu B, Yucesan E. Microencapsulation for Clinical Applications and Transplantation by Using Different Alginates. IntechOpen; 2021.4. [CrossRef]
4. Lu Q, Yang Z, Meng X, Yue Y, Ahmad MA, Zhang W, et al. A Review on Encapsulation Technology from Organic Light Emitting Diodes to Organic and Perovskite Solar Cells. Adv Funct Mater 2021; 31: 2100151. [CrossRef]
5. Güven ŞY. Biyouyumluluk Ve Biyomalzemelerin Seçimi. Suleyman Demirel University Journal of Engineering Sciences and Design 2014; 2(3): 303-11.
6. Carvalho DN, Inácio AR, Sousa RO, Reis RL, Silva TH. Chapter 18 - Seaweed polysaccharides as sustainable building blocks for biomaterials in tissue engineering. In: Torres MD, Kraan S, Dominguez H, editors. Sustainable Seaweed Technologies: Elsevier; 2020. p. 543-87. [CrossRef]
7. Omami M, McGarrigle JJ, Reedy M, Isa D, Ghani S, Marchese E, et al. Islet Microencapsulation: Strategies and Clinical Status in Diabetes. Curr Diab Rep 2017; 17(7): 47. [CrossRef]
8. Woodhams L, Al-Salami H. The roles of bile acids and applications of microencapsulation technology in treating Type 1 diabetes mellitus. Ther Deliv 2017; 8(6): 401-9. [CrossRef]
9. Yücesan E, Göncü B, Başoğlu H, Özten Kandaş N, Kanımdan E, Akbaş F, Yeliz Ersoy E, Ayşan E. Experimental Alternative Method for the Treatment of Hypothyroidism: Capsulation of the Thyroid Tissue. Akdeniz Med J 2018; 5(3): 417-23. [CrossRef]
10. Hasse C, Zielke A, Klock G, Schlosser A, Barth P, Zimmermann U, et al. Amitogenic alginates: key to first clinical application of microencapsulation technology. World J Surg 1998; 22(7): 659-65. [CrossRef]
11. Yucesan E, Basoglu H, Goncu B, Akbas F, Ersoy YE, Aysan E. Microencapsulated parathyroid allotransplantation in the omental tissue. Artif Organs 2019; 43(10): 1022-7. [CrossRef]
12. Aysan E, Kilic U, Gok O, Altug B, Ercan C, Kesgin Toka C, et al. Parathyroid Allotransplant for Persistent Hypocalcaemia: A New Technique Involving Short-Term Culture. Exp Clin Transplant 2016; 14(2): 238-41.
13. Aysan E, Altug B, Ercan C, Kesgin Toka C, Idiz UO, Muslumanoglu M. Parathyroid Allotransplant With a New Technique: A Prospective Clinical Trial. Exp Clin Transplant 2016; 14(4): 431-5.
14. Brown WH. I. Parathyroid Implantation in the Treatment of Tetania Parathyreopriva. Ann Surg 1911; 53(3): 305-17. [CrossRef]
15. Hasse C, Zielke A, Klock G, Barth P, Schlosser A, Zimmermann U, et al. First successful xenotransplantation of microencapsulated human parathyroid tissue in experimental hypoparathyroidism: long-term function without immunosuppression. J Microencapsul 1997; 14(5): 617-26. [CrossRef]
16. Yucesan E, Goncu B, Basoglu H, Ozten Kandas N, Ersoy YE, Akbas F, et al. Fresh tissue parathyroid allotransplantation with short-term immunosuppression: 1-year follow-up. Clin Transplant 2017; 31(11). [CrossRef]
17. de Vos P, Lazarjani HA, Poncelet D, Faas MM. Polymers in cell encapsulation from an enveloped cell perspective. Adv Drug Deliv Rev 2014; 67-68: 15-34. [CrossRef]
18. Poojari R, Srivastava R. Composite alginate microspheres as the next-generation egg-box carriers for biomacromolecules delivery. Expert Opin Drug Deliv 2013; 10(8): 1061-76. [CrossRef]
19. Lopes M, Abrahim B, Veiga F, Seiça R, Cabral LM, Arnaud P, et al. Preparation methods and applications behind alginate-based particles. Expert Opin Drug Deliv 2017; 14(6): 769-82. [CrossRef]
20. Hasse C, Schrezenmeir J, Stinner B, Schark C, Wagner PK, Neumann K, et al. Successful allotransplantation of microencapsulated parathyroids in rats. World J Surg 1994; 18(4): 630-4. [CrossRef]
21. Hasse C, Klock G, Zielke A, Schlosser A, Barth P, Zimmermann U, et al. Transplantation of parathyroid tissue in experimental hypoparathyroidism: *in vitro* and *in vivo* function of parathyroid tissue microencapsulated with a novel amitogenic alginate. Int J Artif Organs 1996; 19(12): 735-41. [CrossRef]
22. Hasse C, Klock G, Schlosser A, Zimmermann U, Rothmund M. Parathyroid allotransplantation without immunosuppression. Lancet 1997; 350(9087): 1296-7. [CrossRef]
23. Hasse C, Schlosser A, Klock G, Barth P, Stinner B, Zimmermann U, et al. [Heterologous transplantation of human parathyroid glands after microencapsulation with clinically suitable alginate : long-term function without immunosuppression in the animal model]. Langenbecks Arch Chir Suppl Kongressbd 1998; 115(Suppl I): 713-8.
24. Hasse C, Bohrer T, Barth P, Stinner B, Cohen R, Cramer H, et al. Parathyroid xenotransplantation without immunosuppression in experimental hypoparathyroidism: long-term in vivo function following microencapsulation with a clinically suitable alginate. World J Surg 2000; 24(11): 1361-6. [CrossRef]
25. Hasse C, Brune M, Lorenz W, Barth P, Metzler W, Breves G, et al. Functional parameters before and after parathyroidectomy: a prospective, randomized long-term trial on different rat strains. Exp Clin Endocrinol Diabetes 2004; 112(7): 364-72. [CrossRef]
26. Zimmermann U, Cramer H, Jork A, Thürmer F, Zimmermann H, Fuhr G, et al. Microencapsulation-Based Cell Therapy. Biotechnology 2001: 547-71. [CrossRef]
27. Tibell A, Rafael E, Wennberg L, Nordenstrom J, Bergstrom M, Geller RL, et al. Survival of macroencapsulated allogeneic parathyroid tissue one year after transplantation in nonimmunosuppressed humans. Cell Transplant 2001; 10(7): 591-9. [CrossRef]
28. Ulrich F, Klupp J, Thürmer F, Rayes N, Seehofer D, Tullius S, et al. Allotransplantation of Encapsulated Human Parathyroid Tissue in Patients With Permanent Hypoparathyroidism. Transplantation 2004; 78: 79. [CrossRef]
29. Cabane P, Gac P, Amat J, Pineda P, Rossi R, Caviedes R, et al. Allotransplant of microencapsulated parathyroid tissue in severe postsurgical hypoparathyroidism: a case report. Transplant Proc 2009; 41(9): 3879-83. [CrossRef]
30. Khryshchanovich V, Ghoussein Y. Allotransplantation of macroencapsulated parathyroid cells as a treatment of severe postsurgical hypoparathyroidism: case report. Ann Saudi Med 2016; 36(2): 143-7. [CrossRef]

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In silico Analysis of Virulence, Resistance Genes and Phylogeny of *Helicobacter pylori* Strains from Different Continents

Farklı Kıtalardaki *Helicobacter pylori* Suşlarının Virülans, Direnç Genleri ve Filogenisinin *in silico* Analizi

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ABSTRACT

Objective: *Helicobacter pylori (H. pylori)* is a bacterium that infects the gastric mucosa of 50% of the world population. It is known that different regional treatment practices used against the infections of *H. pylori* affect both the expression of virulence and antimicrobial resistance genes, giving the bacteria geographic differentiation. The aim of this study was to perform *in silico* analysis of virulence, resistance genes and phylogeny of *H. pylori* strains obtained from people living in different continents.

Material and Method: Complete gene sequences of 18 *H. pylori* strains from six continents were downloaded from the National Center for Biotechnology Information (NCBI) database. The phylogeny of the strains, resistance and virulence genes were analyzed by CSI phylogeny, CARD and VFanalyzer, respectively.

Results: African strains were the most distant identity to European strains. A2147G single nucleotide polymorphism associated with clarithromycin resistance was detected in South American and Asian origin. It was determined that strains were differentiated by a total of 95 related virulence genes under eight headings. The *cagA*, *cagE*, *cagL* and *vacA* genes were found in all strains in Asia.

Conclusion: In conclusion, our study demonstrated that *H. pylori* strains, whose data were collected in different continents, differ from each other in terms of similarities and there is a serious difference especially in terms of virulence genes.

Keywords: *Helicobacter pylori*, virulence genes, *in silico* analysis, geographic phylogeny

ÖZ

Amaç: *Helicobacter pylori (H. pylori)*, tüm dünya popülasyonunun %50'sinin mide mukozasını enfekte eden bir bakteridir ve bölgesel farklı tedavi uygulamaları, hem virülans genleri, hemde antimikrobiyal direnç genlerini etkileyerek, bakteriye coğrafik olarak farklılaşma kazandırdığı bilinmektedir. Çalışmamızda, dünyanın farklı kıtalarında yaşayan insanlardan elde edilen *H. pylori* kökenlerinin filogeni, virülans ve antimikrobiyal direnç genleri açısından *in silico* analizinin yapılması amaçlanmıştır.

Gereç ve Yöntem: Altı kıtadan, toplam 18 *H. pylori* kökenine ait tüm genom dizileri NCBI veritabanından indirilerek çalışmamıza dahil edildi. Kökenlerin evrimsel yakınlıkları, direnç gen belirteçleri ve virülans genleri, sırasıyla CSI filogeni, CARD ve VFanalyzer online yazılımları ile gerçekleştirildi.

Bulgular: Avrupa kökenine göre en uzak benzerlik Afrika kökenleriydi. Klaritromisin direnci ile ilişkili A2147G tek nokta polimorfizmi Güney Amerika ve Asya kökeninde saptandı. Suşların 8 başlık altında toplam 95 ilişkili virülans geni taşıdığı belirlendi. Asya'daki tüm suşlarda *cagA*, *cagE*, *cagL* ve *vacA* genleri bulundu.

Sonuç: Sonuç olarak, çalışmamızın verileri, farklı kıtalarda tespit edilen *H. pylori* kökenlerinin birbirinden farklılıklar gösterdiği ve özellikle virülans genleri açısından ciddi farklılık içerdiğini ortaya koymuştur.

Anahtar Kelimeler: *Helicobacter pylori*, virülans genleri, *in silico* analiz, coğrafik filogeni

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INTRODUCTION

Until Warren and Marshall discovered and identified *Helicobacter pylori* (*H. pylori*) infection in the gastric mucosa in 1983, it was believed that the stomach was sterile due to its highly acidic content (1). However, it is now known that *H. pylori,* which is a spiral-shaped, gram-negative, microaerophilic bacterium, infects the gastric mucosa of 50% of the world population and may lead to gastritis, ulcer, or gastric cancer (2). *H. pylori* infections, which have the ability to colonize the human gastric mucosa, are usually acquired in the early stages of life and can survive for a lifetime (3). The manifestation of *H. pylori* infections in various clinical presentations is due to bacterial virulence factors (e.g. *cagA, vacA, babA*), host genetic characteristics (e.g. age, immune system) and environmental factors (e.g. nutrition, geographical region, living and socio-economic conditions). It has been shown that *H. pylori* may fail to colonize the gastric mucosa by silencing genes that express virulence factors such as flagella, urease production or chemotaxis (4,5). It is understood that different regional treatment algorithms used against *H. pylori* give the bacteria geographic differentiation by affecting both virulence genes and antimicrobial resistance genes (3,4,6,7). The high throughput data obtained by molecular-based systems such as the next generation sequencing systems that have been developed in recent years are important in terms of revealing this geographic differentiation. In addition, by sequencing the whole genome of microbial agents using these systems, virulence factors and resistance genes can be detected quickly and accurately (8). Thus, in our study, we aimed to perform *in silico* analysis of virulence, resistance genes and phylogeny of *H. pylori* strains obtained from people living in different continents.

MATERIAL AND METHOD

Genomic data belonging to a total of 18 *H. pylori* strains from six different continents whose gene sequences uploaded to open databases were downloaded from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) database and included in our study. NCBI accession numbers and information of these strains obtained from patients after endoscopy in Africa, South America, North America, Asia, Europe, and Australia, three from each continent, are presented in Table 1. The strains included in this study, constitute the most selected reference strains in many publications. In addition, although the NCBI database contains data on more than 2000 strains, the whole genome sequencing of all these strains has not yet been completed, but the whole genome sequencing of all the strains we have included in our study has been completed.

Table 1. NCBI accession numbers and information of all strains included in this study.

Continent	Country	Name of the Strain	NCBI Accession number
Africa1	Gambia	Gambia94/24	NC_017371.1
Africa2	South Africa	South Africa7	NC_017361.1
Africa3	South Africa	South Africa20	CP006691.1
S.America1	Venezuela	v225d	NC_017355.1
S. America2	Peru	PeCan18	NC_017742.1
S. America3	El Salvador	ELS37	NC_017063.1
N. America1	Canada	Aklavik117	NC_019560.1
N. America2	USA	J99	NZ_CP011330.1
N. America3	Mexico	29CaP	NZ_CP012907.1
Asia1	India	India7	NC_017372.1
Asia2	Taiwan	ML3	NZ_AP014712.1
Asia3	China	XZ274	NC_017926.1
Europe1	England	ATCC 26695	NC_000915.1
Europe2	France	B38	NC_012973.1
Europe3	Germany	P12	NC_011498.1
Australia1	Australia	ATCC 43504	NZ_LS483488.1
Australia2	Australia	BM012B	NZ_CP007605.1
Australia3	Australia	BM012S	NC_022911.1

S. America: South America, N. America: North America

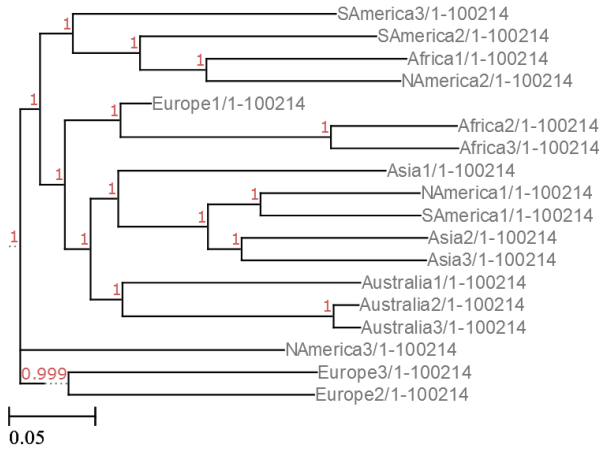

Figure 1. Phylogeny analysis of genomic data of the strains included in the study. S. America: South America, N. America: North America

The evolutionary relatedness of these strains with each other was revealed with the CSI phylogeny software (https://www.genomicepidemiology.org/) (9). The presence of antimicrobial resistance markers in the strains was detected by Comprehensive Antibiotic Resistance Database (CARD - https://card.mcmaster.ca/home) online software (10). Comparative genomic analysis of virulence genes was performed with the VF analyzer software (http://www.mgc.ac.cn/VFs/) (11).

RESULTS

When the identities of 18 *H. pylori* strains from six continents in our study were evaluated *in silico* phylogenetically, the most distant identity to the European1 strain was the strain of Africa2, North America1 and Africa3, and the identity rates were 86.5%, 86.86% and 86.90%, respectively. The strains with the closest identity to the European1 strain were Europe3 with 94.04% and Australia3 with 92.74% identity rates (Table 2).

Table 2: Identitiy rates of all strains examined in this study and the distribution of antimicrobials affected according to resistance gene analysis.

Continent	Country	Strain name	NCBI Accession number	Identity rate	Affected antimicrobials	Resistance gene and SNP
Africa1	Gambia	Gambia94/24	NC_017371.1	92.26%		
Africa2	South Africa	South Africa7	NC_017361.1	86.50%		
Africa3	South Africa	South Africa20	CP006691.1	86.90%		
S. America1	Venezuela	v225d	NC_017355.1	89.61%		
S. America2	Peru	PeCan18	NC_017742.1	91.51%	Clarithromycin	23S rRNA -A2147G
S. America3	El Salvador	ELS37	NC_017063.1	91.48%		
N. America1	Canada	Aklavik117	NC_019560.1	86.86%		
N. America2	USA	J99	NZ_CP011330.1	90.92%		
N. America3	Mexico	29CaP	NZ_CP012907.1	90.51%		
Asia1	India	India7	NC_017372.1	91.18%		
Asia2	Taiwan	ML3	NZ_AP014712.1	90.24%	Clarithromycin	23S rRNA -A2147G
Asia3	China	XZ274	NC_017926.1	91.12%		
Europe1	England	ATCC 26695	NC_000915.1	100%	Quinolone, tetracycline, nitrosamide	HP1181
Europe2	France	B38	NC_012973.1	90.06%		
Europe3	Germany	P12	NC_011498.1	94.04%		
Australia1	Australia	ATCC 43504	NZ_LS483488.1	92.35%		
Australia2	Australia	BM012B	NZ_CP007605.1	92.55%		
Australia3	Australia	BM012S	NC_022911.1	92.74%		

S. America: South America, N. America: North America

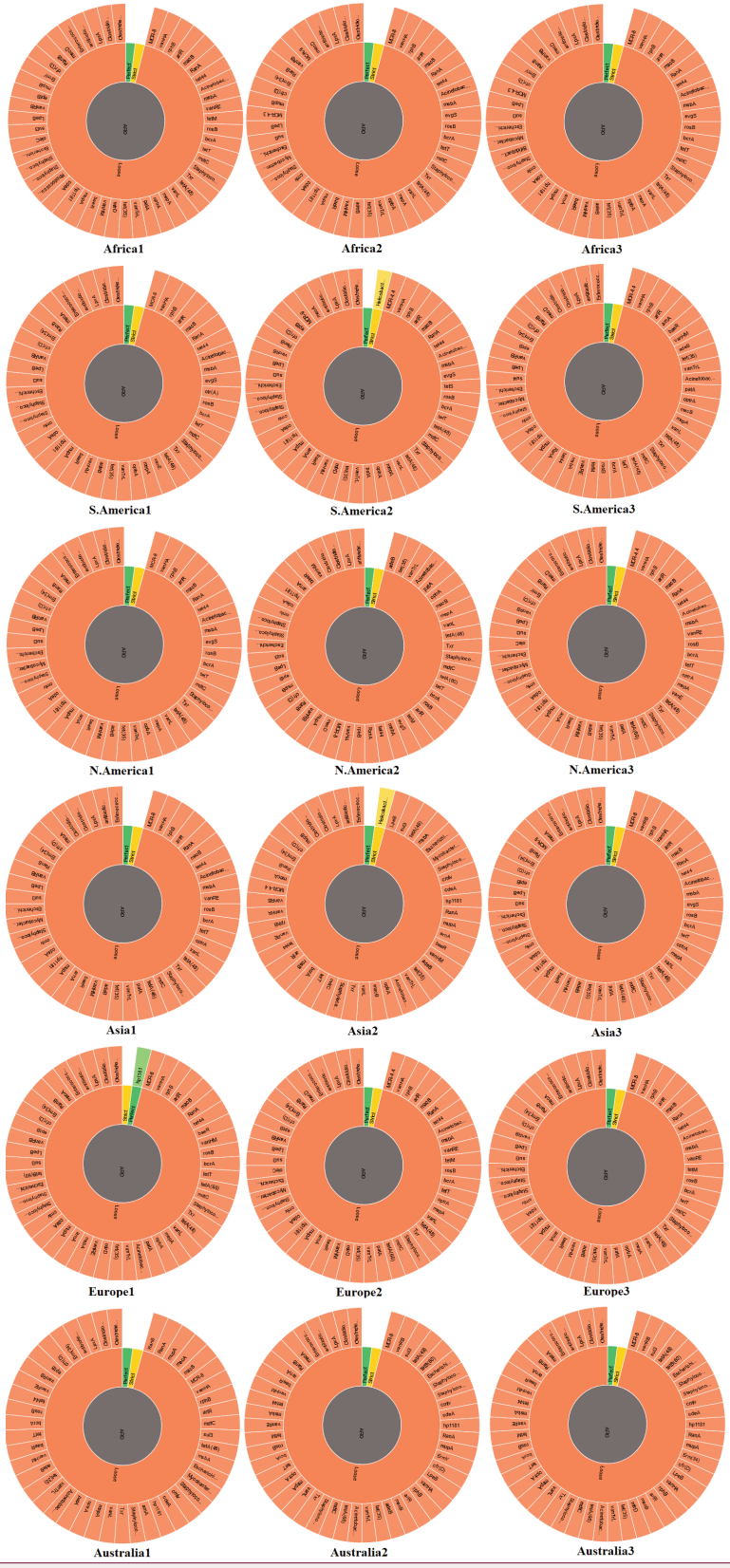

Figure 2. Distribution of resistance gene identifiers of strains included in this study (Green: Perfect Sequence hit, Red: Strict Sequence Hit and Yellow: Loose Sequence Hit). S. America: South America, N. America: North America

In silico analysis of resistance gene identifiers of the genomic data examined in our study is presented in Table 1 and Figure 2. While resistance to clarithromycin was found strict hit to South America2 and Asia2 strains, A2147G single nucleotide polymorphism (SNP) was detected in both strains related to clarithromycin resistance in 23S rRNA gene region. In Europe1, the HP1181 gene, responsible for the expression of resistance to quinolone, tetracycline, and nitrosamine demonstrated a perfect hit. In the genomic data of all strains, loose binding was found against different antibiotic groups at similar levels (Table 2).

According to the *in silico* comparative virulence analysis of the genomic data of the strains examined in our study, it was determined that the virulence of the strains were directed by a total of 95 related virulence genes under eight headings, including acid resistance, adherence, immune evasion, immune modulator, motility, secretion system, toxin and other factors. In the strains belonging to the Australian continent, *H. pylori* strains carried 90.1% of these 95 genes containing open reading frames (ORF) for the virulence genes. In the strains belonging to the North American continent, this rate was the lowest with 68.6% compared to the belonging to other continents. The samples taken from all continents, virulence genes belonging to seven ORF appeared active for acid resistance genes. Moreover, among the adhesins, differences were detected in the number of ORF that would carry the virulence genes of sialic acid-binding adhesins *sabA / hopP, sabB / hopT* and the adhesins that would bind blood group antigens, *babA / hopS and babB / hopT*. Strains carrying ORF for the sialic acid binding adhesin virulence factors, especially in Africa, Asia and North America were found to contain lower ORF counts than the other continents. When the ORF that would carry *futA, futB and futC* virulence genes from Lipopolysaccharide Lewis antigens to immune evasion were analyzed, it was found that their numbers were low in African and European strains. The immunomodulator and virulence factors involved in motility were similar between the strains from different continents. A similarity was found in the strains between the continents in the *napA* gene, which forms the neutrophil-activating protein with an immune modulator effect. In addition to this, the plasticity region was determined as the other virulence factor with the greatest difference in terms of the number of open reading frames in the strains obtained from different continents. The numbers of these regions were the highest in Australian and African strains. It was observed that virulence genes belonging to the cagPAI type secretion system contained less ORF in strains belonging to Africa and North America while it was the highest in the strains of Australia. It was observed that genes belonging to the *cagA, cagE and cagL* ORF, which are virulence genes of the secretion system, were found in all Australian, South American and Asian strains. When the ORF in association with toxin production were examined, it was found that vacuolated cytotoxin-generating ORF were lowest in Australian strains and in all strains of Europe, America, and Asia carried the *vacA* gene (Table 3). When the geographical distribution of virulence genes, which are constantly examined in previous studies such as *cagA*, *cagE, cagL and vacA*, and it was found that the strains in Australia, Asia and South America often carried ORFs for *cagA*, *cagE* and *cagL* genes. However, the lowest level of virulence genes associated with *vacA* toxin production gene was detected in strains belonging to Australia (Table 4).

DISCUSSION

Although half of the world population is colonized by *H. pylori* in their gastric mucosa, it is known that only 15% of them encounter infections, among which only 1% have severe conditions such as gastric cancer. The reason for this situation is explained as a result of the multifactorial nature of the infection (12). Compared to the low prevalence rate of *H. pylori* in geographical regions such as North America and Australia, this rate is higher in Africa and Asia. However, despite the high prevalence of *H. pylori* in Africa and Asia, the rates of gastric cancer formation do not correlate with these results. This remarkable situation has been defined as the "Asian and African enigma". The host's genetic and immune responses, virulence factors of different *H. pylori* strains, and environmental factors are used to explain this enigma (13).

Phuc et al., reported that Asian strains were mostly similar to European strains in their study conducted in Vietnam in 2021 (14). Delahay et al., in their phylogeny geographic study on *H. pylori* strains in 2018, reported that North American strains were distant from Asian strains in terms of similarity and formed a separate cluster, also they added that European and African strains formed a separate cluster from Asian strains (12). In our study, similar to the data of these two studies, we found that European strains were similar to Australian and Asian strains, while they had the lowest similarity to the strains of Africa. It has been concluded that the variations might differ depending on the genes for which the similarities of the strains are examined. Qumar et al., reported that the strains originating from Bangladesh showed 90%-92% similarity with the European strains, in their study conducted in 2021 (15). In our study, we also found that the Asian1 strain which is originating from India was similar to European strains.

Qumar et al. compared the virulence genes of strains obtained from different continents in 2021 and they reported that cagPAI virulence genes could be encoded in the genome in 18 of 20 strains originating from Bangladesh. Moreover, 10 of these 18 strains were found similar to Asian sources while eight of them resembled European sources. They reported that 90% of genes that cause virulence factors were found in their strains originating from Bangladesh (16). Similarly, in the virulence analysis of the genomic data of our isolates originating from Asia, we found this rate at the level of 89%.

Saribasak et al. stated that Asian strains have certain types of *cagA* in their study (16). In our study, we found that the presence of the *cagA* gene appears to be low in the strains belonging to North America, Africa and Europe, that supports this data.

Table 3. Distribution of comparative virulence genes analysis of all strains included in the study.

Virulence Factor	Related gene number	Africa	S. America	N. America	Asia	Europe	Australia
Acid Resistance (1 item)							
Urease	7	7	7	7	7	7	7
Adherence (8 Items)							
Blood group antigen binding adhesins	2	2	2	1.6	2	1.6	2
Sialic acid binding adhesins	2	1	1.6	1	1	1.3	2
HopZ	1	1	1	1	1	1	1
adherence-associated lipoprotein AlpA (hopC)	1	1	1	1	1	1	1
AlpB (hopB)	1	1	1	1	1	1	1
H. pylori adhesin A	1	1	1	1	1	1	1
HorB	1	1	1	1	1	1	1
PEB1	1	-	-	-	-	-	-
Immune evasion (1 Item)							
Lipopolysaccharide Lewis antigens	3	2.3	2.6	2.6	2.6	2.3	0.3
Immune modulator (2 Items)							
Neutrophil-activating protein (HP-NAP)	1	1	1	1	1	1	1
Outer inflammatory protein	1	1	1	0.6	1	1	1
Motility (1 Item)							
Flagella	38	36.6	37	37	36.6	36.6	37
Others (2 Items)							
DupA (duodenal ulcer promoting)	1	-	-	-	-	-	-
Plasticity region	3	2	0.3	1	1.6	1	.2.6
Secretion system (2 Items)							
Cag PAI type IV secretion system	26	8	23.6	8	23.6	16.3	24
T4SS effectors cytotoxin-associated gene A	1	0.3	1	0.3	1	0.6	1
Toxin (2 Items)							
Vacuolating cytotoxin	1	0.6	1	1	1	1	0.3
Cytolethal distending toxin	3	-	-	-	-	-	-
Total ORF gene counts (Mean)	95	65.9	82.8	65.2	83.4	74.1	85.6
Total gene percentage (%)		69.3	87.1	68.6	89.8	78	90.1

S. America: South America, N. America: North America

Yamaoka et al. reported that geographic differences in gastric cancer cases depend not only on the differences of *cagA* and *vacA*, but also on the differences in other virulence factors such as *oipA* and *babA* (17). In our study, we determined that there are differences in virulence genes such as *oipA* and *babA*, as well as differences in *cagA* and *vacA*. Erzin et al., in their study on Turkish patients in 2006, reported that *cagE* was an independent variable for duodenal ulcer and gastric cancer. They

Table 4. The status of the frequently studied virulence genes of the strains included in the study.

Related virulence factor	related gene	Africa	S. America	N. America	Asia	Europe	Australia
Adherence	babA/hopS	1.33	1.00	1.33	1.00	1.67	2.33
Adherence	babB/hopT	1.00	1.00	0.67	1.00	1.67	1.00
Adherence	sabA/hopP	1.33	1.00	0.67	1.33	0.67	1.00
Adherence	sabB/hopO	0.00	0.67	0.33	0.00	1.00	1.00
Immune evasion	futA	0.33	0.67	0.33	0.33	0.33	1.00
Immune evasion	futB	1.33	1.00	1.67	1.33	1.67	1.00
Immune modulator	oipA/hopH	1.00	1.33	0.67	1.67	1.00	1.00
Other	Plasticity region	2.00	0.33	1.00	1.67	1.00	2.67
Cag PAI type IV secretion system	cagA	0.33	1.67	0.33	1.00	0.67	1.00
Cag PAI type IV secretion system	cagE	0.33	1.00	0.33	1.00	0.67	1.00
Cag PAI type IV secretion system	cagL	0.33	1.00	0.33	1.00	0.67	1.00
Toxin	vacA	0.67	1.00	1.00	1.00	1.00	0.33

S. America: South America, N. America: North America

found *cagE* and *vacA* as biomarkers in duodenal ulcer patients and *cagE* and *babA2* as biomarkers in gastric cancer patients (18). Erzin et al, in 2008, examined host factors and bacterial virulence factors in their study, emphasized the importance of *babA2* in terms of cancer development and also determined the protective role of IL-1B 31TT genotype in the host (19).

While our *cagE* data may explain the low incidence of gastric cancer in Africa, similar to the results of this study, it contradicts the situation in Asia. Hence the *cagE* or *babA2* alone might not be the solution to the African-Asian enigma. Demiryas et al., in their study conducted in 2020, reported that when they compared the virulence genes of *H. pylori* strains found in gastric cancer, duodenal ulcer and non-ulcer dyspepsia patient groups, *cagL* was significantly different between the groups (20). The inclusion of different virulence factors in all these studies indicates that our knowledge about *H. pylori*'s direction of cancer development is limited. Kocazeybek et al., in 2015, showed that not only the presence of *cagA* but also special motifs or patterns such as EPIYA seen in the *cagA* region could cause geographical differences in this carcinogenesis process (21). In a study conducted in 2020, Saribas et al., showed that gastrointestinal diseases can be explained not only by EPIYA patterns but also by including host genetic factors such as HLAs (22). Kocak et al., reported that in patients with *cagL* and *cagA* positive *H. pylori*, bacterial virulence factors, as well as host genetic factors such as HLAs, are also involved in the gastrointestinal disease process (23). Like Erzin et al., (19), Sarıbaş et al., (22) and Kocak et al., (23) emphasized the importance of host factors in the process. It has strengthened our belief that our knowledge of the mechanism of cancer development of *H. pylori* is lacking. On the other hand, Sun et al., in their study in 2020, regarding the virB11 protein produced by the *virB11* gene located in the *H. pylori* plasticy region, found that this protein plays a role in the ATP formation mechanism of *H. pylori* and is important for providing energy (24). Yamaoka showed the protective effect of the plasticity region against gastric cancer (25). Besides Africa and Asia strains, as well as Australia strains, the amount of ORF plasticity region carriage was found high in our study, so this virulence gene alone cannot explain the Africa-Asian enigma.

In the study Mwangi et al., conducted in 2020, they stated that the *H. pylori* strain they isolated from a patient in Kenya was similar to Asian stains. Although the main virulence factors they detected were found in other African strains, it was reported that this strain was not similar to other African strains in terms of phylogeny (26). In our study, we found that our results were similar in terms of virulence factors, ORF and phylogenies, but that African strains we studied generally differed from European and Asian strains in terms of both virulence gene prevalence and phylogeny. Lamichhane et al., reported that they found subgroups of Australian strains belonging to people coming from Europe 200 years ago, and the strains they examined were similar to Australian strains (27). Among the strains we examined in our study, we found that Australian strains have more similarities to European strains than other continents.

As Suzuki et al., pointed out, the geographic differences of *H. pylori* strains may reveal human migrations. They also noted that the genetic diversity within *H. pylori* strains was much greater than in other bacterial strains and 50 times greater than in human populations. In addition, they reported that with the molecular epidemiological studies carried out with new molecular sequencing techniques, we can have more information about

both virulence genes and resistance genes, and the mechanisms of gastric cancer formation can thus be understood (28).

Boyanova et al. reported that more than 20% of clarithromycin resistance was seen in Asia, Europe and South America, and above 10% of quinolone resistance was seen in Asia (29). In our study, we detected clarithromycin resistance and A2147G SNP in 23S rRNA gene causing this resistance in an Asian and a South American strain. However, we found a gene associated with quinolone resistance only in our European strain, this could be related to the limited number of strains that we included in this study. Kocazeybek and Tokman reported that primary antibiotic resistance detected in *H. pylori* strains were affected by geographic differences and crowded population (3). This is consistent with our resistance data.

The limitations of our study were both not using the genomic data of all strains in the NCBI database and not having our own strains in the study. The strains we included in our study appear to be mostly selected reference strains in many publications. In addition, although the NCBI database contains data on more than 2000 strains, the whole genome sequencing of all these strains has not yet been completed, but the whole genome sequencing of all the strains we have included in our study has been completed.

CONCLUSIONS

In conclusion, our study has revealed that *H. pylori* strains, whose data were reported from different continents, vary from each other phylogenetically and especially in terms of virulence genes. We believe that it is important to reveal the virulence genes of these strains by using new molecular sequencing techniques, in order to reveal the situation described as both the African-Asian enigma and the pathogenesis mechanisms used by *H. pylori* during gastric cancer.

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REFERENCES

1. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet 1984; 1(8390): 1311-5. [CrossRef]
2. Keikha M, Karbalaei M. Correlation between the geographical origin of Helicobacter pylori homB-positive strains and their clinical outcomes: a systematic review and meta-analysis. BMC Gastroenterol 2021; 21: 181. [CrossRef]
3. Kocazeybek B, Tokman HB. Prevalence of Primary Antimicrobial Resistance of H. pylori in Turkey: A Systematic Review. Helicobacter 2016; 21: 251-60. [CrossRef]
4. Chang WL, Yeh YC, Sheu BS. The impacts of H. pylori virulence factors on the development of gastroduodenal diseases. J Biomed Sci 2018; 25: 68. [CrossRef]
5. de Brito BB, da Silva FAF, Soares AS, Pereira VA, Santos MLC, Sampaio MM, et al. Pathogenesis and clinical management of Helicobacter pylori gastric infection. World J Gastroenterol 2019; 25: 5578-89. [CrossRef]
6. Thyagarajan SP, Ray P, Das BK, Ayyagari A, Khan AA, Dharmalingam S, et al. Geographical difference in antimicrobial resistance pattern of Helicobacter pylori clinical isolates from Indian patients: Multicentric study. J Gastroenterol Hepatol 2003; 18: 1373-8. [CrossRef]
7. Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. Helicobacter pylori virulence and genetic geography. Science 1999; 284(5418): 1328-33. [CrossRef]
8. Gilchrist CA, Turner SD, Riley MF, Petri WA Jr, Hewlett EL. Whole-genome sequencing in outbreak analysis. Clin Microbiol Rev. 2015; 28: 541-63. [CrossRef]
9. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLoS One 2014; 9: e104984. [CrossRef]
10. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, et al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 2020; 48: D517-D525. [CrossRef]
11. Liu B, Zheng D, Jin Q, Chen L, Yang J. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. Nucleic Acids Res 2019; 47(D1): D687-D692. [CrossRef]
12. Delahay RM, Croxall NJ, Stephens AD. Phylogeographic diversity and mosaicism of the Helicobacter pylori tfs integrative and conjugative elements. Mob DNA 2018; 9: 5. [CrossRef]
13. Ofori EG, Adinortey CA, Bockarie AS, Kyei F, Tagoe EA, Adinortey MB. Helicobacter pylori Infection, Virulence Genes' Distribution and Accompanying Clinical Outcomes: The West Africa Situation. Biomed Res Int 2019; 2019: 7312908. [CrossRef]
14. Phuc BH, Tuan VP, Dung HDQ, Binh TT, Tung PH, Tri TD, et al. Helicobacter pylori type 4 secretion systems as gastroduodenal disease markers. Sci Rep 2021; 11: 4584. [CrossRef]
15. Qumar S, Nguyen TH, Nahar S, Sarker N, Baker S, Bulach D, et al. A comparative whole genome analysis of Helicobacter pylori from a human dense South Asian setting. Helicobacter 2021; 26: e12766. [CrossRef]

16. Saribasak H, Salih BA, Yamaoka Y, Sander E. Analysis of Helicobacter pylori genotypes and correlation with clinical outcome in Turkey. J Clin Microbiol 2004; 42(4): 1648-51. [CrossRef]
17. Yamaoka Y, Kato M, Asaka M. Geographic differences in gastric cancer incidence can be explained by differences between Helicobacter pylori strains. Intern Med 2008; 47: 1077-1083. [CrossRef]
18. Erzin Y, Koksal V, Altun S, Dobrucali A, Aslan M, Erdamar S, et al. Prevalence of Helicobacter pylori vacA, cagA, cagE, iceA, babA2 genotypes and correlation with clinical outcome in Turkish patients with dyspepsia. Helicobacter 2006; 11: 574-80. [CrossRef]
19. Erzin Y, Koksal V, Altun S, Dobrucali A, Aslan M, Erdamar S, et al. Role of host interleukin 1beta gene (IL-1B) and interleukin 1 receptor antagonist gene (IL-1RN) polymorphisms in clinical outcomes in Helicobacter pylori-positive Turkish patients with dyspepsia. J Gastroenterol 2008; 43(9): 705-10. [CrossRef]
20. Demiryas S, Caliskan R, Saribas S, Akkus S, Gareayaghi N, Kirmusaoglu S, et al. The association between cagL and cagA, vacAs-m, babA genes in patients with gastric cancer, duodenal ulcer, and non-ulcer dyspepsia related to Helicobacter pylori. Acta Gastroenterol Belg 2020; 83(3): 385-92.
21. Kocazeybek BS, Caliskan R, Erdamar Cetin S, Ergin S, Kuskucu M, Kepil N, et al. Patterns of EPIYA motifs among cagA-positive Helicobacter pylori strains: a case-control study in a Turkish population with Eurasian geographical features. J Med Microbiol 2015; 64(10): 1117-23. [CrossRef]
22. Saribas S, Demiryas S, Yilmaz E, Uysal O, Kepil N, Demirci M, et al. Association between human leukocyte antigen gene polymorphisms and multiple EPIYA-C repeats in gastrointestinal disorders. World J Gastroenterol 2020; 26: 4817-32. [CrossRef]
23. Kocak BT, Saribas S, Demiryas S, Yilmaz E, Uysal O, Kepil N, et al. Association between polymorphisms in HLA-A, HLA-B, HLA-DR, and DQ genes from gastric cancer and duodenal ulcer patients and cagL among cagA-positive Helicobacter pylori strains: The first study in a Turkish population. Infect Genet Evol 2020; 82: 104288. [CrossRef]
24. Sun DL, Gong ZH, Shao SL, Shi XL, Yuan XY, Luo H, et al. virB11 gene potentially involves in ATP metabolism to provide energy in H. pylori infection. Microb Pathog 2020; 142: 104067. [CrossRef]
25. Yamaoka Y. Roles of the plasticity regions of Helicobacter pylori in gastroduodenal pathogenesis. J Med Microbiol 2008; 57: 545-53. [CrossRef]
26. Mwangi C, Njoroge S, Tshibangu-Kabamba E, Moloo Z, Rajula A, Devani S, et al. Whole Genome Sequencing Reveals Virulence Potentials of Helicobacter pylori Strain KE21 Isolated from a Kenyan Patient with Gastric Signet Ring Cell Carcinoma. Toxins (Basel) 2020; 12: 556. [CrossRef]
27. Lamichhane B, Wise MJ, Chua EG, Marshall BJ, Tay CY. A novel taxon selection method, aimed at minimizing recombination, clarifies the discovery of a new sub-population of Helicobacter pylori from Australia. Evol Appl 2019; 13: 278-89. [CrossRef]
28. Suzuki R, Shiota S, Yamaoka Y. Molecular epidemiology, population genetics, and pathogenic role of Helicobacter pylori. Infect Genet Evol 2012; 12(2): 203-13. [CrossRef]
29. Boyanova L, Mitov I. Geographic map and evolution of primary Helicobacter pylori resistance to antibacterial agents. Expert Rev Anti Infect Ther 2010; 8(1): 59-70. [CrossRef]

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Intracytoplasmic Sperm Injection (ICSI) in B6D2F1 and CB6F1 Strains Mice Using Cauda Epididymal Spermatozoa

B6D2F1 ve CB6F1 Irk Farelerde Kauda Epididimal Spermatozoa Kullanılarak İntrastoplazmik Sperm Enjeksiyonu (ICSI)

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ABSTRACT

Objective: Reproductive biotechnology studies focus on the long-term storage of embryos (cryopreservation), embryo cultures, genome editing of embryos and embryo transfer. Micromanipulation techniques in reproduction biotechnologies have an important role, especially in studies investigating assisted reproductive technology in laboratory animals. The aim of the present study was to investigate the effect of epididymal spermatozoa injected to oocyte by intracytoplasmic sperm injection (ICSI) in different mice strains. In this study, we evaluated the *in vitro* development of post-ICSI derived embryos using cauda epididymal sperm.

Material and Method: Female mice (8-10 weeks) were superovulated using pregnant mare serum gonadotropin/human chorionic gonadotropin (PMSG/hCG) and ~14h post hCG, the mice were sacrificed, and the oocytes were collected. Spermatozoa from the cauda epididymal of a 12-week-old were used on the same strain for ICSI and the *in vitro* developmental potential was evaluated. Finally, the embryos were cultured for 120 hours at 5% CO_2 with 37°C.

Results: The results showed that the two-cell embryo of the B6D2F1 strain (79.31%) was significantly higher than the CB6F1 (56.26%) ($p<0.05$). While the blastocyst rate was comparable between both the B6D2F1 strain (68.75%) and CB6F1 strain (69.57%) ($p>0.05$).

Conclusion: ICSI using cauda epididymal sperm is a suitable application for *in vitro* embryo development in B6D2F1 and CB6F1 strains. Finally, ICSI success of the B6D2F1 mice strains was found to be higher than CB6F1 mice strains.

Keywords: Mouse, oocyte, ICSI, development

ÖZ

Amaç: Üreme biyoteknolojisi alanındaki çalışmalar; embriyoların uzun süre saklanması (kriyoprezervasyon), embriyo kültürü, embriyoların genom düzenlenmesi araştırmaları ve embriyo transferi gibi konular üzerinde yoğunlaşmaktadır. Üreme biyoteknolojilerinde mikromanipülasyon teknikleri, özellikle laboratuvar hayvanlarında yardımcı üreme teknolojisinin araştırıldığı çalışmalarda önemli bir yere sahiptir. Bu çalışmanın amacı, farklı fare ırklarında intrastoplazmik sperm enjeksiyonu (ICSI) ile epididimal spermatozoanın oosite enjeksiyonunu araştırmaktır. Bu çalışmada, kauda epididimal fare spermi kullanılarak yapılan ICSI uygulaması sonrasında elde edilen embriyoların *in vitro* gelişimi değerlendirilmiştir.

Gereç ve Yöntem: Dişi fareler (8-10 hafta), gebe kısrak serum gonadotropini/insan koryonik gonadotropini (PMSG/hCG) kullanılarak süperovüle edilmiş, hCG'den ~14 saat sonra fareler sakrifiye edilerek oositler toplanmıştır. 12 haftalık erkek farenin kauda epididiminden alınan spermatozoa, aynı ırk oositle ICSI için kullanılmış ve *in vitro* gelişim potansiyeli değerlendirilmiştir. Son olarak tüm embriyolar 120 saat süre ile %5 CO_2 ve 37°C'de kültüre edilmiştir.

Bulgular: Sonuçlar, B6D2F1 (%79,31) ırkının 2 hücreli embriyo gelişiminin CB6F1 (%56,26) ırklı farelerdeki 2 hücreli embriyo gelişiminden önemli ölçüde yüksek olduğunu göstermiştir ($p<0,05$). Blastosist oranı B6D2F1 (%68,75) ve CB6F1 (%69,57) ırkları arasında karşılaştırılmıştır ($p>0,05$).

Sonuç: B6D2F1 ve CB6F1 fare ırklarında, kauda epididimal sperma kullanılarak yapılan ICSI *in vitro* embriyo gelişimi için uygun bir yöntemdir. Sonuç olarak, B6D2F1 farelerde ICSI'nın başarısı, CB6F1 ırk farelere göre daha yüksektir.

Anahtar Kelimeler: Fare, oosit, ICSI, gelişim

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INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is the name given to the method of injecting spermatozoa into the oocytes through microsurgical methods. It is a common approach in clinical practice in the treatment of infertility problems with various causes, and in the production of farm and laboratory animals (transgenic and low-reproductive strains) (1,2). There is a need for a convenient and cost-effective method for long-term sperm preservation of transgenic mice (3).

It is well known that the genetic background of mouse strains affects morphological parameters of sperm (4-6) and *in vitro* produced embryo development (7). Most common mouse strains are used for developing new transgenic lines. And their embryo development results can be compared to the other strains (4-6). Furthermore, using different mouse strains in ICSI can affect embryo development (8-10).

The genetic characteristics of different mouse strains affect embryo development results in mouse embryo studies. Also, no research was found in literature about results of *in vitro* developments of ICSI between oocytes from CB6F1 mouse strain. The present study analyzes the *in vitro* development rates of embryos produced by the application of ICSI in comparison to the mouse strains of CB6F1 (C57BL/6J x Balb/c) and B6D2F1 (DBA2 x C57BL/6J).

MATERIAL AND METHOD

All mice experiments were approved by Koc University Local Ethics Committee for Animal Experiments (approval number: 2014-05). The animals were kept in Koc University, Animal Research Facility of Center for Translational Medicine (KUTTAM) under 12 hours light - 12 hours dark cycle, and a diet of commercial pellet food ad-libitum and automatic water containers were provided according to the Guide for the Care and Use of Laboratory Animals.

Superovulation and Oocyte Collection

10 IU of pregnant mare serum gonadotropin (Sigma G4877-PMSG) was injected by intraperitoneal (IP) to the female mice between 17:00 and 18:00, and 48 hours later, 10 IU of human chorionic gonadotropin (Organon-hCG) was injected by intraperitoneally between 17:00 and 18:00 hours. Fourteen hours after the hCG injection, female mice were sacrificed, and the oocytes were collected by rupturing the oviductal ampullae. The oocytes were washed in HEPES buffered human tubal fluid (HTF) medium with hyaluronidase, and then washed three times in a hepes buffered-M2 medium supplemented with 4 mg/ml BSA (Sigma Catalog, No A-3311, Fraction V). The culture media containing the collected oocytes were kept in an incubator at 37ºC and in 5% CO_2 for 30–60 minutes until the ICSI was performed (10). 10 µl of embryo culture drops were added to a petri dish and covered with mineral oil (Lifeguard-Life Global) in order to avoid the contamination, evaporation and to ensure integrity of the drops. The petri dishes were then placed in an incubator at 37°C, in 5% CO_2 and humidity for gassing at least 2 hours prior to embryo collection.

Preparation of Spermatozoa

The male mice of each strain were sacrificed, cauda epididymis were removed and incubated in 500 µl embryo culture medium (LifeGlobal Media, LGGG-020) at 37°C, 5% CO_2 for 10 minutes. Then, 100 µl was collected from the top of the tube and transferred to the cryotube. The spermatozoa were rapidly frozen in liquid nitrogen which lead to breakage of sperm tail and head (11,12).

Intracytoplasmic Sperm Injection (ICSI)

After oocyte collection from the petri dish, a 10 cm petri dish cover was used, the area of which was divided into four sections. Fifteen µl drops were placed in the petri dish and covered with 7-8 ml of mineral oil (Figure 1), and 3 µl of frozen-thawed spermatozoa were placed in M2+3% polyvinyl-pyrrolidone (PVP) drops. ICSI working plate prepared with small droplet of manipulation HEPES buffered HTF medium. The sperm was mixed with five volumes of a 10% solution of PVP (v/v) in HEPES buffered HTF medium. A droplet of piezo pipette prepared for washing in volumes of a 3% solution of PVP in HEPES buffered HTF medium. The spermatozoa were mixed using a manipulation pipette to ensure individual cell formation. ICSI started 16-18 hours after hCG injection and performed by using a Nikon Eclipse and an inverted microscope equipped with Eppendorf TransferMan/Nk 2 micromanipulators. An Eppendorf CellTram Vario embryo holding pipette console and an Eppendorf embryo holding pipette with a 35° angle, 15 µm inner diameter and a 100 µm outer diameter were mounted on the left micromanipulator. An Eppendorf CellTram Air embryo manipulation pipette console and an Eppendorf embryo ICSI piezo drill micropipette (Origio/CooperSurgical, U.S.A, Piezo-6-25) with a 6° angle, 6 µm inner diameter and an 8 µm outer diameter were mounted on the right micromanipulator. The spermatozoa were taken sequentially into the injection pipette (Figure 2A). The injection pipette was transferred into manipulation drops. Using a holding pipette (Optimas, reference code: OMH1202030) with loaded 2-3 µl mercury, the oocytes were fixed at the 6 or 12 o'clock position of the MII (Figure 2A). Using a piezo drill (Prime Tech PMM4G) injection pipette, a single-pulse piezo of 4/4 (intensity/velocity) was made to puncture the zona pellucida of the oocyte (Figure 2B, 2C). Minimum suction of cytoplasm was applied into the ICSI injection pipette (Figure 2D, 2E) and a single-pulse piezo of 2/1 (intensity/velocity) was applied to break the cytoplasm and inject the sperm to oocyte immediately (Figure 2F, 2G). The sperm injection into the cytoplasm was conducted at room temperature (17-18°C) (11,12).

Post-ICSI Embryo Culture

The ICSI-treated oocytes were transferred into a culture medium, and the embryos were washed in at least three times and then incubated at 37º C and in 5% CO_2 for 96–120 hours respectively. The two cell and blastocyst developments were evaluated on 2th day and 5th day, respectively.

Statistical Assessment of Results

IBM SPSS Statistics for Windows (Version 24.0. Armonk, NY: IBM Corp.) was used for the statistical assessment of the results with

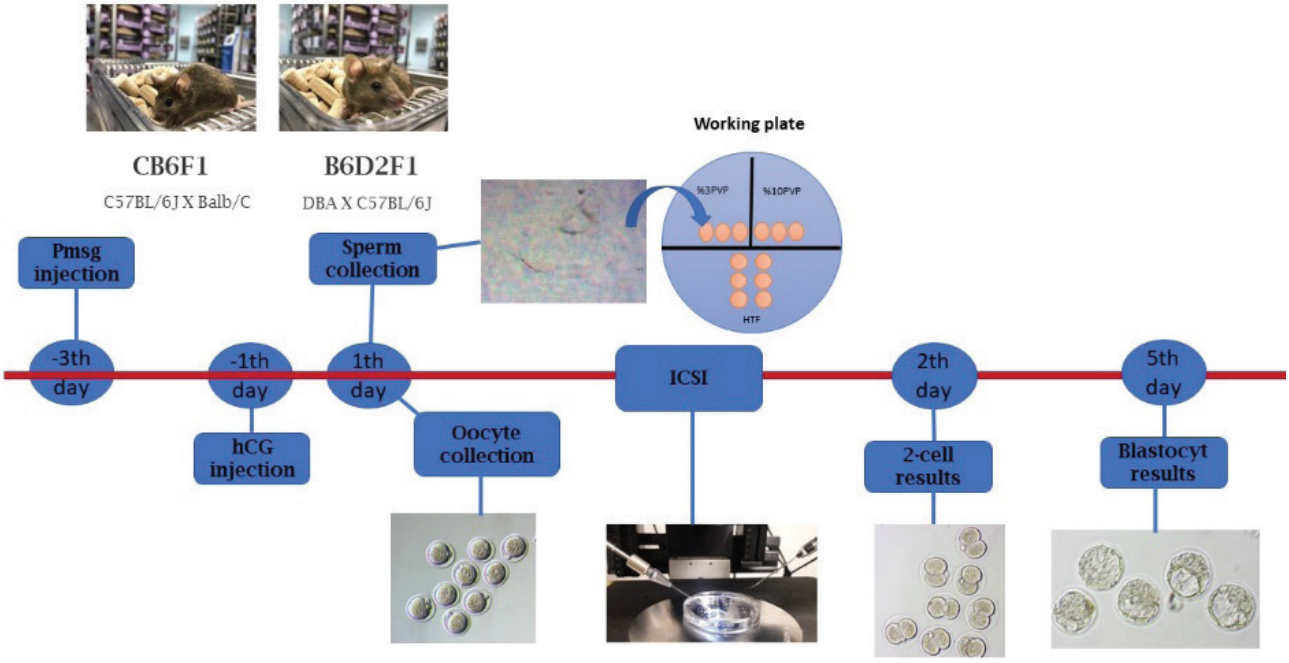

Figure 1. Schematic depicting the experimental timeline in mouse intrastoplasmic sperm injection (ICSI).

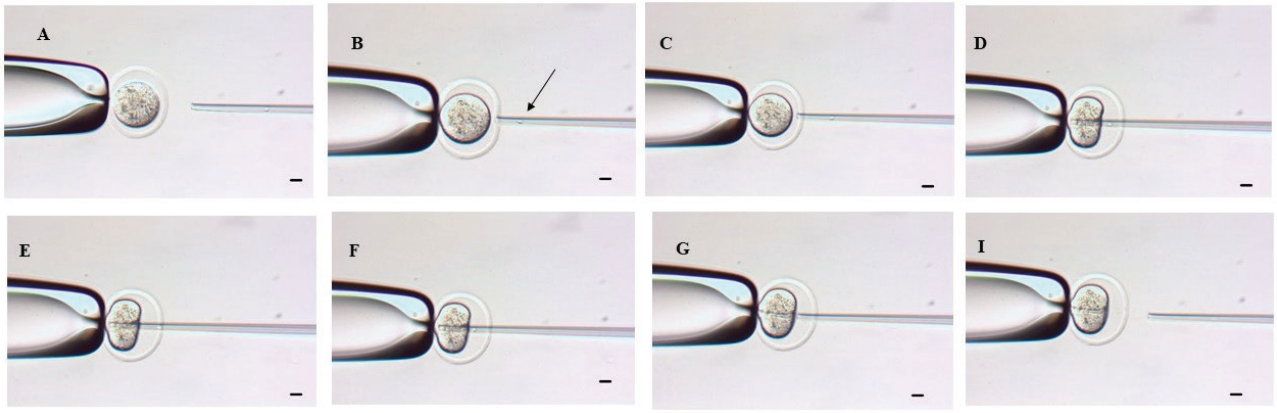

Figure 2. Piezo drill of ICSI on mouse oocyte. (A) The sperm was sucked into the injection pipette, (B) the arrow is sperm in injection pipette and zona pellucida aspirated by injection pipet, (C) a single-pulse piezo was made to puncture the zona pellucida of the oocyte, (D,E) aspiration of cytoplasm is applied into injection pipette and apply a single-pulse piezo to break to cytoplasm, (F) the sperm injected to oocyte, (G,I) when withdrawing the ICSI pipette out of the sperm injected mouse oocyte (20*X*).

sufficient number of repetitions. ANOVA and Chi-square tests were used for the analysis of variance and differences between groups. The p-values <0.05 were considered statistically significant. All experiments were replicated three times.

RESULTS

According to development evaluations done after *in vitro* culture, the two cell development rates were 56.26% and 79.31%, in CB6F1 and B6D2F1 strain groups, respectively p<0.05, Table 1). The differences of two cell development were found significantly important between in CB6F1 and B6D2F1 strain groups (p< 0.05). The blastocyst development rates were 68.75% and 69.57%, in CB6F1 and B6D2F1 strain groups, respectively (Table 1). The differences of blastocyst development were no found significantly between the CB6F1 and B6D2F1 strain groups (p> 0.05, Table 1).

Table 1. *In vitro* development of post-ICSI derived embryos from different mouse strains.

Strain	Number of oocytes	Number of 2-cell embryos (%)	Number of blastocyst from 2-cell stage embryo (%)
CB6F1	27	16[a] (56.26±11.33)	11[a] (68.75±29.46)
B6D2F1	29	23[b] (79.31±16.16)	16[a] (69.57±8.42)

(a,b)-Differences between the same columns with different symbols (a, b) were found to be significant ($p<0.05$). There was significant difference in B6D2F1 and CB6F1 of two cell development rates.

(a)-Same characters (a) in the same column are not significant ($p>0.05$). There was no significant difference in B6D2F1 and CB6F1 of blastocyst development rates.

DISCUSSION

The mice are appropriate in research of ICSI-derived embryo development for improving of laboratory animal reproductive technologies. ICSI is widely used in both animals and humans as an effective approach to study the fertility (13). Mice are considered as model animals in medical research and ICSI are used to better understand the biology of fertilization (14). It has been shown that both cauda and caput epididymal spermatozoa were used in different mouse strains (13). The aim of this research is to investigate the rates of *in vitro* development of embryos produced by ICSI to different mouse strains. This present study has investigated the rate of *in vitro* culture development in ICSI-derived embryos in CB6F1 (C57BL/6J x Balb/c) and B6D2F1 (DBA2 x C57BL/6J) mice by cauda epididymal spermatozoa.

It is increasingly recognized that ICSI is affected by both age and subspecies, as the study of BALB/c mice demonstrates a relationship between ICSI results and species difference (15). Also, Ogonuki et al. reported about animal strains were significant effect on two-cell embryo development in ICSI (16). The present study also establishes a statistically significant difference in the rates of two-cell embryo development, and we observed strain affect in this study.

In FVB and CD-1 mice, two cell developments are around 80% as a result of piezo ICSI with cauda and epididymal sperm (11). Likewise, in our study, similarly, two-cell development in B6D2F1 mouse race was around 80%. On the other hand, CB6F1 race had a lower rate of two cell development.

Compared intracytoplasmic sperm injections in inbred (C57BL/6) and hybrid mice (B6C3F1 and B6D2F1), and although the fertilization and embryo development rates were found to be similar in the C57BL/6 and hybrid strains, the post-implantation rates were poorer in the C57BL/6 strains than in the other strains, which was believed to be linked to sperm factors (17).

The other study discussed that the reproductive potential of caput epididymal spermatozoa and carried out comparative ICSI applications with caput and cauda spermatozoa in FVB and CD1 strains. Following these applications, the authors established a blastocyst development rate of 57% with caput sperms and 63% with cauda sperms (18).

Mallol et al. reported that blastocyst development rate of 57% in mouse embryos following an ICSI application after a freezing-thawing protocol in hybrid B6CBAF1 (C57Bl/6xCBA/J) mice (12). Moreira et al. reported that achieved a blastocyst development rate of 60% with ICSI application (19). The results of the present study were compared with those of the two commonly used strains, revealing that the two-cell development rate of the B6D2F1 strain was significantly higher, and the blastocyst development rate of the CB6F1 strain, especially after two-cell development, was also significantly higher.

We compared *in vitro* development of ICSI derived embryos with cauda epididymal sperm in two different mouse strains. The two-cell development were found significantly important between in CB6F1 and B6D2F1 strain groups ($p<0.05$). While the blastocyst developments were not found significantly important between CB6F1 and B6D2F1 strain groups ($p>0.05$, Table 1).

Future studies can focus on comparing strains of ICSI derived embryos; *in vivo* development, cryopreservation and embryonic gene expression levels. Based on the results of the present study, a useful model can be developed for veterinary and laboratory animal sciences. Also, these findings of ICSI-derived embryos with biotechnological methods would contribute to the improvement and development of the national livestock. The result of this study showed that ICSI with cauda epididymal sperm is a suitable application for embryo development in both mouse strains. In conclusion, because of its ease of use, this ICSI technique is considered useful in laboratory animals.

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REFERENCES

1. Andersen ML, Winter LM. Animal models in biological and biomedical research-experimental and ethical concerns. An Acad Bras Cienc 2019; 91. [CrossRef]
2. Yanagimachi R. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. Reprod Biomed Online 2005; 10(2): 247-88. [CrossRef]
3. Li MW, Willis BJ, Griffey SM, Spearow JL, Lloyd KC K. Assessment of three generations of mice derived by ICSI using freeze-dried sperm. Zygote 2009; 17(3): 239-51. [CrossRef]
4. Songsasen N, Leibo SP. Cryopreservation of mouse spermatozoa II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. Cryobiology 1997; 35: 255-69. [CrossRef]
5. Sztein JM, Farley JS, Mobraaten LE. In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 2000; 63: 1774-80. [CrossRef]
6. Nishizono H, Shioda M, Takeo T, Irie T, Nakagata N. Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. Biol of Reprod 2004; 71: 973-8. [CrossRef]
7. Dandekar PV, Glass RH. Development of mouse embryos in vitro is affected by strain and culture medium. Gamete Res 1978; 17: 279-85. [CrossRef]
8. Ogonuki N, Mori M, Shinmen A, Inoue K, Mochida K, Ohta A, et al. The effect on intracytoplasmic sperm injection outcome of genotype, male germ cell stage and freeze-thawing in mice. PLoS One 2010; 5(6): e11062. [CrossRef]
9. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394, 369-74. [CrossRef]
10. Taskin AC, Kocabay A, Gül S, Caglar-Erkal K. Comparison of parthenogenetic oocyte activation in different mouse strains on in vitro development rate and quality. Vet Hekim Der Derg 2021; 92(1): 42-8.
11. Wang Y, Yamauchi Y, Wang Z, Zheng H, Yanagimachi R, Ward MA, et al. Both Cauda and Caput Epididymal Sperm Are Capable of Supporting Full-Term Development in FVB and CD-1 Mice. Dev Cell 2020; 55(6): 675-6. [CrossRef]
12. Mallol A, Santaló J, Ibáñez E. Psammaplin a improves development and quality of somatic cell nuclear transfer mouse embryos. Cell Reprogram 2014;16(5): 392-406. [CrossRef]
13. Fernández-González R, Laguna R, Ramos-Ibeas P, Pericuesta E, Alcalde-Lopez V, Perez-Cerezales S, et al. Successful ICSI in Mice Using Caput Epididymal Spermatozoa. Front Cell Dev Biol 2019; 7: 346. [CrossRef]
14. Stein P and Schultz RM. ICSI in the mouse. Methods Enzymol 2010; 476: 251-62. [CrossRef]
15. Ohta H, Sakaide Y and Wakayama T. Age and substrain-dependent sperm abnormalities in BALB/c mice and functional assessment of abnormal sperm by ICSI. Hum Reprod 2009; 24(4): 775-81. [CrossRef]
16. Ogonuki N, Mori M, Shinmen A, Inoue K, Mochida K, Ohta A, et al. The effect on intracytoplasmic sperm injection outcome of genotype, male germ cell stage and freeze-thawing in mice. PLoS One, 2010; 5(6): e11062. [CrossRef]
17. Kawase Y, Iwata T, Toyoda Y, Wakayama T, Yanagimachi R and Suzuki H. Comparison of intracytoplasmic sperm injection for inbred and hybrid mice. Mol Reprod Dev 2001; 60(1): 74-8. [CrossRef]
18. Fernández-González R, Laguna R, Ramos-Ibeas P, Pericuesta E, Alcalde-Lopez V, Perez-Cerezales S, et al. Successful ICSI in Mice Using Caput Epididymal Spermatozoa. Front Cell Dev Biol 2019; 7: 346. [CrossRef]
19. Moreira PN, Giraldo P, Cozar P, Pozueta J, Jiménez A, Montoliu L, et al. Efficient generation of transgenic mice with intact yeast artificial chromosomes by intracytoplasmic sperm injection. Biol Reprod 2004; 71(6): 1943-7. [CrossRef]

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Differential Expression of *LEF1* Isoforms in Adult Lymphoid and Myeloid Malignancies

Yetişkin Lenfoid ve Miyeloid Malignitelerde *LEF1* İzoformlarının Farklı Ekspresyonu

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ABSTRACT

Objective: Lymphoid enhancer-binding factor-1 (LEF1) is one of the key regulators of lymphocyte proliferation and its aberrant expression is a prognostic factor for lymphoid or myeloid malignancies. In this study, we focused on the expression of *LEF1* isoforms in several hematological malignancies and found tissue-specific differential expression for the full-length (FL)-*LEF1* gene and its tumor suppressor (Δ*LEF1*) variant.

Material and Method: Fifty-three leukemia/lymphoma patients were included in this study. Diagnostic samples of "lymphoid group" patients: Chronic Lymphoblastic Leukemia (CLL) (n=10), B-cell Acute Lymphoblastic Leukemia (B-ALL) (n=9) and "myeloid group" patients: Chronic Myeloblastic Leukemia (CML) (n=12), Acute Myeloid Leukemia (AML) (n=13), and Multiple Myeloma (MM) (n=9) were studied. Healthy bone marrow, peripheral blood cells, and CD34 positive cells were used as controls. Total (T) and *FL-LEF1* transcript levels were examined by using quantitative real-time polymerase chain reaction (qRT-PCR). T and *FL-LEF1* mRNA ratios were also evaluated for calculation of Δ*LEF1*.

Results: *LEF1* levels were significantly high in lymphoid malignancies, but MM and AML patients have decreased *LEF1* levels. Although CLL patients have high *FL-LEF1* levels, the ratio of the T/FL levels was significantly decreased.

Conclusion: *LEF1* is a proliferation factor for lymphocytes and not only its differential overexpression but also the ratio of T/FL isoforms seem to accompany leukemia progress.

Keywords: *LEF1*, alternative splicing, lymphoid, myeloid, leukemia

ÖZ

Amaç: Lenfositlerin çoğalmasındaki önemli düzenleyicilerden biri olan lenfoid güçlendirici bağlama faktörü-1 (LEF1)'in anormal ekspresyonu, lenfoid veya miyeloid maligniteler için prognostik bir faktördür. Bu çalışmada, farklı *LEF1* izoformlarının çeşitli hematolojik malignitelerdeki ekspresyonu incelenmiş ve tüm uzunluktaki *LEF1* (*FL-LEF1*) anlatımı ile tümör baskılayıcı özelliğe sahip kısa izoformu (Δ*LEF1*) için dokuya özgü farklılıklar tespit edilmiştir.

Gereç ve Yöntem: Çalışmaya 53 yetişkin lösemi/lenfoma hastasının tanı anı örnekleri dahil edilmiştir. Çalışmaya dahil edilen hastalar, "lenfoid grubu"; Kronik Lenfoblastik Lösemi (KLL) (n=10), B hücreli Akut Lenfoblastik Lösemi (B-ALL) (n=9) ve "miyeloid grubu"; Kronik Miyeloblastik Lösemi (KML) (n=12), Akut Miyeloid Lösemi (AML) (n=13) ve Multipl Miyelom (MM) (n=9) gruplarından oluşmaktadır. Sağlıklı kemik iliği, periferik kan hücreleri ve CD34 pozitif hücreler kontrol olarak kullanılmıştır. Total (T) ve FL-LEF1 transkript seviyeleri, gerçek zamanlı kantitatif polimeraz zincir reaksiyonu (qRT-PZR) ile incelenmiştir. T ve FL-LEF1 oranları da ΔLEF1 hesaplaması için değerlendirilmiştir.

Bulgular: *LEF1*'in lenfoid malignitelerde anlamlı derecede yüksek olduğu, MM ve AML hastalarında ise *LEF1* seviyelerinde azalma olduğu görülmüştür. KLL hastalarında *FL-LEF1* seviyeleri yüksek olmasına rağmen, *T/FL-LEF1* seviyelerinin önemli ölçüde azaldığı tespit edilmiştir.

Sonuç: *LEF1*, lenfositler için bir çoğalma faktörüdür ve sadece aşırı ekspresyonu değil, aynı zamanda *T/FL-LEF1* izoformlarının oranının da lösemi ilerlemesine eşlik ettiğini düşündürmektedir..

Anahtar Kelimeler: *LEF1*, alternatif kırpılma, lenfoid, miyeloid, lösemi

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INTRODUCTION

Lymphoid enhancer binding factor-1 (LEF1), a member of the regulatory proteins called high mobility group proteins, is one of the key regulators in the proliferation of lymphocytes (1,2). *LEF1* is a downstream target of the WNT pathway involved in regulating cellular proliferation, differentiation, and organ development (3). Deregulated *LEF1* expression was observed in specific cancer types such as colon and prostate cancer or various hematological malignancies like acute lymphoblastic leukemia (ALL), Burkitt lymphoma (BL), and Chronic Lymphocytic Leukemia (CLL) (4-6).

LEF1 has different isoforms caused by alternative splicing and these alternative isoforms can change transcriptional activity (7). The full-length *LEF1* (*FL-LEF1*) isoform derives from selective activation of a promoter that can bind to beta catenin, and the other short isoform of *LEF1 (ΔLEF1)*, located within the intronic promoter of *LEF1* and acts as a dominant-negative isoform (8). *FL-LEF1* is expressed in mitotically active cells and behaves as a growth-promoting isoform; *ΔLEF1* uses an alternative translation start site and lacks exons 1 and 2, resulting from the loss of the beta catenin binding site and acts as an inhibitory isoform and suppresses cell growth (9). Different *LEF1* isoforms are transcribed in harmony under normal circumstances. Studies have shown that aberrant *LEF1* activity and differential expression patterns in different *LEF1* isoforms were important in leukemia development (6, 10). *FL-LEF1* was shown as a pro-survival factor in B-cell CLL cells, expressing abundant *LEF1*, but the expression of *LEF1* gene is much lower or absent in low-grade B-cell non-Hodgkin's lymphoma (NHL), suggesting differences in the activity of the *LEF* genes/isoforms between different malignancies (11,12).

Our previous study showed that pediatric T-cell and B-cell ALL patients have differential expression of *LEF1* variants (13). Therefore, in this study, we focused on *LEF1* isoforms' expression in a group of adult lymphoid and myeloid hematological malignancies and found tissue-specific differential expression for *LEF1* gene variants.

MATERIAL AND METHOD

Study Population

Fifty-three newly diagnosed patients in the hematology clinics were enrolled in this study. Diagnostic samples of "lymphoid group" patients: CLL (4) (n=10), B-cell ALL (n=9), and "myeloid group" patients: AML (n=13), Chronic Myeloblastic Leukemia (CML) (n=12), and Multiple Myeloma (MM) (n=9) patients were diagnosed according to WHO criteria (14) at Hacettepe University, Faculty of Medicine, Hematology Department. Twenty-six males and twenty-seven females with a mean age of 58.5±13.3 for the lymphoid group and 56.6±14.1 for the myeloid group were selected. Diagnostic samples whose white blood cell (WBC) count was above $20x10^3$/μL were included. Patients who received chemotherapy prior to the study were excluded. The control group comprised of the bone marrow samples of 10 healthy individuals, and the CD34 positive cells of nine healthy donors for allogeneic peripheral stem cell transplantation. Additionally, peripheral blood cells (n=5) were also studied. The study was approved by Hacettepe University Ethics Boards and Commissions (Project number: LUT 10/45) and all patients and healthy individuals signed written informed consent.

RNA Isolation and cDNA Synthesis

Mononuclear cells were collected, and total RNA was isolated according to the manufacturer's instructions (Qiagen, Germany). RNA quantity was measured by Nanodrop (ND-1000, Nanodrop Technologies, Inc., USA). 1000 ng RNA was used for cDNA synthesis by using random primers (20 μM, Roche Diagnostics, Germany).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Analysis

Total (T)-LEF and *FL-LEF1* mRNA levels were determined in patients and controls by qRT-PCR. To measure the balance between the isoforms, the ratio of *T- LEF1/ FL- LEF1* was also calculated for each hematologic malignancy. The primer sequences of *LEF1* isoforms and the qRT-PCR protocol were shown previously (13). *CYPA* and *BACT* genes were used as housekeeping genes. All runs were performed twice and each sample was run in duplicate. qRT-PCR analyses were performed on LightCycler 480 (Roche Diagnostics).

Statistical Analysis

The $2^{-\Delta\Delta Ct}$ method was used for relative quantification (15). Groups were compared by using the Mann-Whitney U test and p-value of ≤ 0.05 was considered significant.

RESULTS

We determined both *LEF1* isoforms' expression in control CD34 positive cells, bone marrow cells, and peripheral blood cells and showed *LEF1* variants at variable levels. Each patient sample was evaluated for *T* and *FL-LEF1* expression levels. *T-LEF1* including long and *ΔLEF1* isoforms and *T/FL LEF1* ratio gives us *ΔLEF1* isoforms levels. The ratio of *total LEF1/ FL LEF1* in the lymphoid group (CLL and ALL) and myeloid group (AML, CML, and MM) patients were also evaluated.

In general analysis, lymphoid group patients had aberrant *FL-LEF1* expression (p=0.01), but T-*LEF1* levels and the T/FL ratio were normal compared to healthy control samples. Myeloid group patients showed decreased T-*LEF1* (p=0.0009) and *FL-LEF1* (p=0.003) expression, but the T/FL ratio was found to be similar compared to the controls. When we compared leukemia groups, the myeloid leukemia patients showed significantly decreased T (p=0.008) and FL-*LEF1* (p=0.004) expression compared to lymphoid leukemia patients, and this deregulated T and FL-*LEF1* expression levels caused decreased T/FL ratio (p=0.03) in these patients.

Although there is no significant difference in T-*LEF1* expression, the oncogenic isoform *FL-LEF1* showed increased expression (p<0.0001) compared to healthy bone marrow and

peripheral blood cells in CLL patients. The ratio of T/FL-*LEF1* was also found to decrease in CLL patients (p=0.002) (Figure 1). Differently from CLL patients, adult B-ALL patients showed increased total (p=0.02) and *FL-LEF1* (p<0.001) expression levels (Figure 1).

T-*LEF-1* and *FL-LEF1* mRNA expression levels were found to be significantly decreased in AML patients compared to CD34 positive healthy controls. In addition, the T/FL ratio was found to be similar when compared to the controls (Figure 2A). CML patients showed slightly decreased *FL-LEF1* expression, but the ratio of T/*FL-LEF1* was similar when compared to the controls (Figure 2).

The total expression levels of the *LEF1* gene and the ratio of *T/FL-LEF1* were found to be decreased in MM samples (p=0.02, p=0.02, respectively) (Figure 2).

DISCUSSION

LEF-1 plays a role in the canonical Wnt signaling pathway by β-catenin and the LEF1/TCF complex, and activation of Wnt-target genes (16). Up-to-date studies point to activation of the

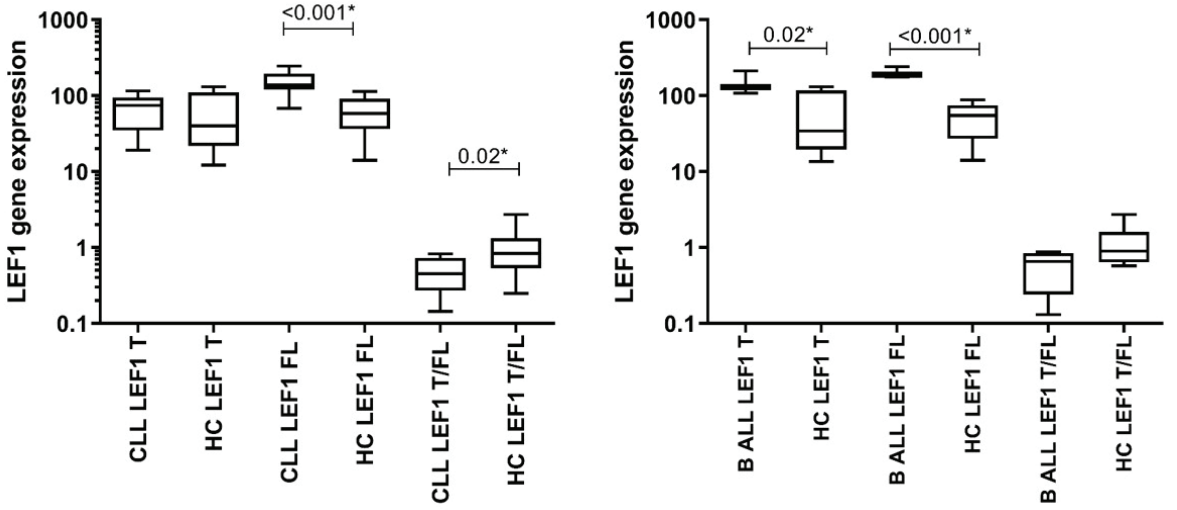

Figure 1. Expression analysis of *LEF1* isoforms in lymphoid group patients. *LEF1* expression in chronic lymphoblastic leukemia and adult B-cell acute lymphoblastic leukemia (B-ALL).

T represents the total expression of *LEF1*, FL represents full-length *LEF1* expression and T/FL represents the total and full-length ratio of *LEF1* expression. T/FL ratio was used for calculating short (ΔLEF1) isoform. CLL: Chronic Lymphoblastic Leukemia; B-ALL: Adult B-cell Acute Lymphoblastic Leukemia, HC: Healthy control sample. Statistical significance is shown by *.

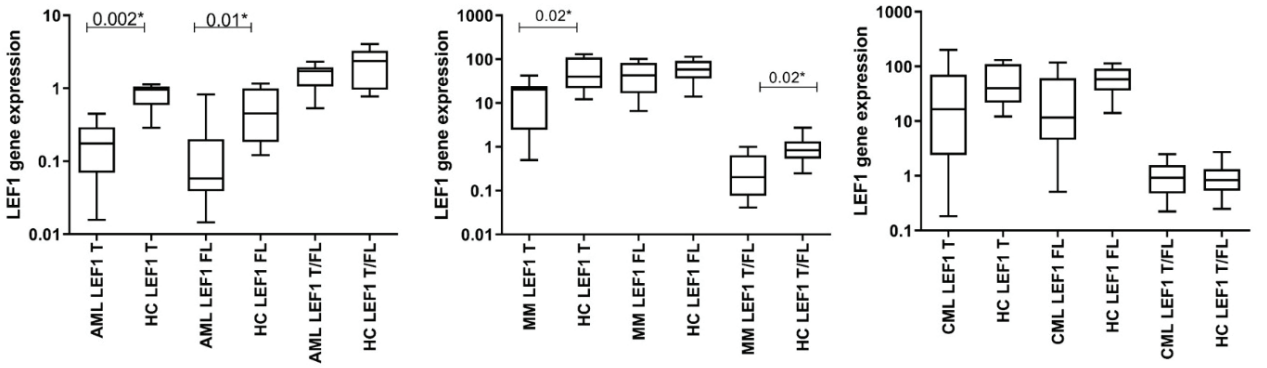

Figure 2. Expression analysis of *LEF1* isoforms in myeloid group and multiple myeloma patients.

T represents the total expression of *LEF1*, FL represents full-length *LEF1* expression and T/FL represents the total and full-length ratio of *LEF1* expression. T/FL ratio was used for calculating short (ΔLEF1) isoform. AML: Acute Myeloid Leukemia; MM: Multiple Myeloma; CML: Chronic Myeloid Leukemia; HC: Healthy control sample. Statistical significance is shown by *.

WNT pathway, and increased *LEF1* expression as being associated with the pathogenesis of different leukemia types (17-21). Not only high *LEF1* expression levels, but the existence of different isoforms and their heterogeneous contribution may also explain its role in malignancy development. The imbalance between the *LEF1* isoforms was shown in the transformation of malignancies (22,23). The role of the *LEF1* gene in cell growth is not restricted upon the WNT pathway; studies have shown that *ΔLEF1* stimulates the TCR alpha enhancer as strongly as *FL-LEF1*, which indicates that beta catenin has independent transactivation of the *LEF1* gene (24). In human CD34 positive cells, inhibition of *FL-LEF1*, but not beta catenin, affects proliferation and apoptosis of these progenitor cell populations, which supports that WNT is an independent function of *LEF1* in early hematopoiesis (25).

In our previous study, we showed that pediatric ALL patients have increased *FL-LEF1* expression and an abnormal ratio of long and short *LEF1* isoforms have been found in B and T cell ALL (13). Here we defined the different expression patterns of *LEF1* isoforms among adult lymphoid and myeloid malignancies and showed that although lymphoid and myeloid malignancies had significantly increased *LEF1* expression, lymphoid group patients have significantly aberrant *LEF1* activity according to the myeloid patients' group. We also further compared the lymphoid and the myeloid cohort and found that myeloid patients (including AML, CML, and MM) have decreased T-*LEF1* expression levels. These results indicate that although imbalanced *LEF1* isoforms were seen in both lymphoid and myeloid series, *LEF1* activation is more related to lymphoid leukemia.

Guiterrez *et. al* reported *LEF-1* expression in B-cell lymphocytosis previously (11). Recently, we have established that *LEF1* is a highly specific marker for the diagnosis of B-CLL (26) and/or *LEF1* expression is related to atypical CLL (27). In our study, CLL cases showed differences in oncogenic *FL-LEF1* and *ΔLEF1* variants leading to the instability between long and short transcript ratio. These findings indicate that *LEF1* oncogenic activity might have a role in CLL but due to the limited number of CLL patients, our results did not show any subtype differences.

Adult B-ALL patients have upregulated total and *FL-LEF1* isoforms, but the T/FL ratio was not changed indicating that the upregulation of T-*LEF1* expression is related to high *FL-LEF1* expression. Kühnl et al., identified that high *LEF1* expression is a prognostic factor for adult B- ALL patients, and high *LEF1* levels were associated with the outcome of the patients (28). While full length *LEF1* acts as an oncogene, a short transcript of the *LEF1* gene functions as a tumor suppressor (29). Our results showed that adult B-ALL patients have increased *FL-LEF1* isoform expression beside high *ΔLEF1* isoform and the balance of T/*FL-LEF1* were not changed.

In addition to the lymphoid group patients, our CML patients have unaffected *LEF1* gene expression and *LEF1* variant ratios. CML is a type of cancer resulting mostly from a reciprocal translocation t(9;22)(q34;q11), which caused the accumulation of an active fusion kinase protein called BCR-ABL. The oncogenic role of BCR-ABL is the dominant causative genetic defect in adult CML patients, but the WNT pathway activity is associated with tyrosine kinase inhibitory resistance and acute blast phase in leukemic stem cells in CML (30). Further studies are needed to understand the role of the *LEF1* gene in CML patients.

Unlike CML patients, multiple myeloma patients have decreased *T-LEF1* expression, resulting in an abnormal T/*FL-LEF1* isoform ratio, but no change in *FL-LEF1* expression. These findings point to a reduced T/*FL-LEF1* ratio occurring by impaired *ΔLEF1* levels. In addition, AML patients showed decreased total and *FL-LEF1* and a slightly decreased level in *ΔLEF1* expression. Recent studies reported that abnormal *ΔLEF1* levels, but normal *FL-LEF1* levels, were found in AML patients (31). Our results showed MM patients have similar results compared to AML patients in the literature.

In conclusion, we reported the *LEF1* isoform levels in adult lymphoid and myeloid leukemia patients and observed impaired full-length or *ΔLEF1* expression. Our results point to aberrant *LEF1* expression is mostly associated with lymphoid leukemia.

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REFERENCES

1. Reya T, O'Riordan M, Okamura R, Devaney E, Willert K, Nusse R, et al. Wnt signaling regulates B lymphocyte proliferation through a LEF-1 dependent mechanism. Immunity 2000; 13: 15-24. [CrossRef]
2. Asally M, Yoneda Y. Beta-catenin can act as a nuclear import receptor for its partner transcription factor, lymphocyte enhancer factor-1 (lef-1). Exp Cell Res 2005; 308: 357-63. [CrossRef]
3. Santiago L, Daniels G, Wang D, Deng FM, Lee P. Wnt signaling pathway protein LEF1 in cancer, as a biomarker for prognosis and a target for treatment. Am J Cancer Res 2017; 7: 1389-406.
4. Sutton LA, Ljungström V, Enjuanes A, Cortese D, Skaftason A, Tausch E, et al. Comparative analysis of targeted next-generation sequencing panels for the detection of gene mutations in chronic lymphocytic leukemia: an ERIC multi-center study. Haematologica 2021; 106: 682-91. [CrossRef]
5. Morgan RG, Ridsdale J, Payne M, Heesom KJ, Wilson MC, Davidson A, et al. LEF-1 drives aberrant beta-catenin nuclear localization in myeloid leukemia cells. Haematologica 2019; 104: 1365-77. [CrossRef]
6. Petropoulos K, Arseni N, Schessl C, Stadler CR, Rawat VP, Deshpande AJ, et al. A novel role for Lef-1, a central transcription mediator of Wnt signaling, in leukemogenesis. J Exp Med 2008; 205: 515-22. [CrossRef]
7. Hovanes K, Li TW, Munguia JE, Truong T, Milovanovic T, Lawrence Marsh J, et al. Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. Nat Genet 2001; 28: 53-7. [CrossRef]
8. Atcha FA, Munguia JE, Li TW, Hovanes K, Waterman ML. A new beta-catenin-dependent activation domain in T cell factor. J Biol Chem 2003; 278: 16169-75. [CrossRef]
9. Hovanes K, Li TW, Waterman ML. The human LEF-1 gene contains a promoter preferentially active in lymphocytes and encodes multiple isoforms derived from alternative splicing. Nucleic Acids Res 2000; 28: 1994-2003. [CrossRef]
10. Metzeler KH, Heilmeier B, Edmaier KE, Rawat VP, Dufour A, Dohner K, et al. High expression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia. Blood 2012; 120: 2118-26. [CrossRef]
11. Gutierrez A, Jr., Tschumper RC, Wu X, Shanafelt TD, Eckel-Passow J, Huddleston PM, 3rd, et al. LEF-1 is a prosurvival factor in chronic lymphocytic leukemia and is expressed in the preleukemic state of monoclonal B-cell lymphocytosis. Blood 2010; 116: 2975-83. [CrossRef]
12. Seke Etet PF, Vecchio L, Nwabo Kamdje AH. Interactions between bone marrow stromal microenvironment and B-chronic lymphocytic leukemia cells: any role for Notch, Wnt and Hh signaling pathways? Cell Signal 2012; 24: 1433-43. [CrossRef]
13. Erbilgin Y, Hatirnaz Ng O, Can I, Firtina S, Kucukcankurt F, Karaman S, et al. Prognostic evidence of LEF1 isoforms in childhood acute lymphoblastic leukemia. Int J Lab Hematol 2021; 43(5): 1093-1103. [CrossRef]
14. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016; 127: 2391-405. [CrossRef]
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-8. [CrossRef]
16. Danek P, Kardosova M, Janeckova L, Karkoulia E, Vanickova K, Fabisik M, et al. beta-Catenin-TCF/LEF signaling promotes steady-state and emergency granulopoiesis via G-CSF receptor upregulation. Blood 2020; 136: 2574-87. [CrossRef]
17. Guo X, Zhang R, Liu J, Li M, Song C, Dovat S, et al. Characterization of LEF1 High Expression and Novel Mutations in Adult Acute Lymphoblastic Leukemia. PLoS One 2015; 10: e0125429. [CrossRef]
18. Lyapichev KA, Sakhdari A, Khoury JD, O'Malley DP, El Hussein S, Yin CC, et al. Lymphoid enhancer binding factor 1 (LEF1) expression is significantly higher in Hodgkin lymphoma associated with Richter syndrome relative to de novo classic Hodgkin lymphoma. Ann Diagn Pathol 2020; 49: 151636. [CrossRef]
19. Xu R, Huang X, Li C, Deng C, Li M, Wu P, et al. Bone marrow mesenchymal stromal cells in chronic myelomonocytic leukaemia: overactivated WNT/beta-catenin signalling by parallel RNA sequencing and dysfunctional phenotypes. Br J Haematol 2021; 193: 928-40. [CrossRef]
20. Ng OH, Erbilgin Y, Firtina S, Celkan T, Karakas Z, Aydogan G, et al. Deregulated WNT signaling in childhood T-cell acute lymphoblastic leukemia. Blood Cancer J 2014; 4: e192. [CrossRef]
21. Fetisov TI, Lesovaya EA, Yakubovskaya MG, Kirsanov KI, Belitsky GA. Alterations in WNT Signaling in Leukemias. Biochemistry (Mosc) 2018; 83: 1448-58. [CrossRef]
22. Jimenez J, Jang GM, Semler BL, Waterman ML. An internal ribosome entry site mediates translation of lymphoid enhancer factor-1. RNA 2005; 11: 1385-99. [CrossRef]
23. Li TW, Ting JH, Yokoyama NN, Bernstein A, van de Wetering M, Waterman ML. Wnt activation and alternative promoter repression of LEF1 in colon cancer. Mol Cell Biol 2006; 26: 5284-99. [CrossRef]
24. Hsu SC, Galceran J, Grosschedl R. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol 1998; 18: 4807-18. [CrossRef]
25. Skokowa J, Cario G, Uenalan M, Schambach A, Germeshausen M, Battmer K, et al. LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med 2006; 12: 1191-7. [CrossRef]
26. Menter T, Trivedi P, Ahmad R, Flora R, Dirnhofer S, Tzankov A, et al. Diagnostic Utility of Lymphoid Enhancer Binding Factor 1 Immunohistochemistry in Small B-Cell Lymphomas. Am J Clin Pathol 2017; 147: 292-300. [CrossRef]
27. Soliman DS, Al-Kuwari E, Siveen KS, Al-Abdulla R, Chandra P, Yassin M, et al. Downregulation of Lymphoid enhancer-binding factor 1 (LEF-1) expression (by immunohistochemistry and/ flow cytometry) in chronic Lymphocytic Leukemia with atypical immunophenotypic and cytologic features. Int J Lab Hematol 2021; 43: 515-25. [CrossRef]
28. Kuhnl A, Gokbuget N, Kaiser M, Schlee C, Stroux A, Burmeister T, et al. Overexpression of LEF1 predicts unfavorable outcome in adult patients with B-precursor acute lymphoblastic leukemia. Blood 2011; 118: 6362-7. [CrossRef]
29. Yokoyama NN, Pate KT, Sprowl S, Waterman ML. A role for YY1 in repression of dominant negative LEF-1 expression in colon cancer. Nucleic Acids Res 2010; 38: 6375-88. [CrossRef]
30. Masamoto Y, Kurokawa M. Targeting chronic myeloid leukemia stem cells: can transcriptional program be a druggable target for cancers? Stem Cell Investig 2018; 5:10. [CrossRef]
31. Feder K, Edmaier-Schroger K, Rawat VPS, Kirsten N, Metzeler K, Kraus JM, et al. Differences in expression and function of LEF1 isoforms in normal versus leukemic hematopoiesis. Leukemia 2020; 34: 1027-37. [CrossRef]

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Romatizmal Kalp Kapak Hastalığı Olan Türk Hastalarda *IL-1Ra* VNTR Gen Polimorfizminin İlişkisi Üzerine Bir Çalışma

A Study on the Relationship of *IL-1Ra* VNTR Gene Polymorphism in Turkish Patients with Rheumatic Heart Disease

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ÖZ

Amaç: Romatizmal kalp kapak hastalığının (RKKH)'da dahil olduğu çeşitli otoimmün ve kronik enflamatuvar hastalıklarda, *IL-1* reseptör antagonisti (*IL-1Ra*) A2 allelinin uzun süreli ve şiddetli proenflamatuvar bağışıklık cevabıyla ilişkili olabileceği yönünde çalışmalar bulunmaktadır. Ancak, RKKH patogenezinde *IL-1Ra* varyantının ilişkisi ile ilgili çalışmalar oldukça sınırlıdır. Bu çalışmanın amacı, Türk hastalarda *IL-1Ra* rs2234663 değişken sayıda ardışık yineleme (VNTR) varyantları ile RKKH duyarlılığı ve/veya hastalığın şiddeti arasında bir ilişki olup olmadığını araştırmaktır.

Gereç ve Yöntem: Bu vaka-kontrol çalışmasında (165 RKKH/300 kontrol) *IL-1Ra* VNTR için polimeraz zincir reaksiyonu (PZR) yöntemi ile genotipleme yapılmıştır.

Bulgular: Hastalar ve kontroller arasında genotip ve allel frekanslarında istatistiksel olarak anlamlı fark tespit edilmiştir (p<0,001). A1 allel frekansı, RKKH hastalarında kontrol grubuyla karşılaştırıldığında, anlamlı olarak daha yüksek bulunmuştur (p=0,004; OR=3,821; %95 GA=1,459-10,005). A3 allel frekansı hasta grubunda kontrol grubuna kıyasla istatistiksel olarak anlamlı şekilde artış göstermiştir (p=0,034; OR=2,536; %95 GA=1,045-6,152). Şiddetli ve hafif kapak hasarı olan hastalar arasında genotip dağılımları istatistiksel olarak anlamlı şekilde farklı bulunmuştur (p=0,031). A2 alleli şiddetli kapak hasarı olan hastalarda hafif kapak hasarı olan hastalara göre istatistiksel olarak yüksek olarak tespit edilmiştir (p=0,003).

Sonuç: *IL-1Ra* VNTR rs2234663 varyantlarının Türk toplumunda RKKH açısından duyarlı bireylerin tanımlanmasında kullanılabilecek uygun bir biyobelirteç olabileceği önerilmektedir.

Anahtar Kelimeler: IL-1Ra, rs2234663, polimorfizm, romatizmal kalp kapak hastalığı, romatizmal ateş

ABSTRACT

Objective: There are studies that suggest that the *IL-1* receptor antagonist (*IL-1Ra*) A2 allele may be associated with a long-term and severe proinflammatory immune response in various autoimmune and chronic inflammatory diseases, including rheumatic valvular heart disease (RVHD). However, studies on the relationship of the *IL-1Ra* variant in the pathogenesis of RVHD are very limited. The aim of this study was to investigate whether there is a relationship between *IL-1Ra* rs2234663 variable number tandem repeat (VNTR) variants and RVHD susceptibility and/or disease severity in Turkish patients.

Material and Method: In this case-control study (165 RVHD/300 controls), we genotyped *IL-1Ra* VNTR using the polymerase chain reaction (PCR) method.

Results: There was a statistically significant difference in genotype and allele frequencies between patients and controls (p<0.001). The A1 allele frequency was found to be significantly higher in RVHD patients compared to the control group (p=0.004; OR=3.821; 95% CI=1.459-10.005). The A3 allele showed a statistically significant increase in the patient group compared to the control group (p=0.034; OR=2.536; 95% CI=1.045-6.152). Genotype distributions were statistically significantly different between patients with severe and mild valve damage (p=0.031). The A2 allele was statistically higher in patients with severe valve damage compared to patients with mild valve damage (p=0.003).

Conclusion: *IL-1Ra* VNTR can be recommended as a suitable biomarker that can be used to identify susceptible individuals in terms of RVHD susceptibility in the Turkish population.

Keywords: IL-1Ra, rs2234663, polymorphism, rheumatic valvular heart disease, rheumatic fever

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GİRİŞ

Romatizmal kalp kapak hastalığı (RKKH) otoimmün bir hastalıktır. Romatizmal ateş (RA), A grubu β-hemolitik streptokokların neden olduğu boğaz enfeksiyonundan sonra antijenler ile konak doku proteinleri arasındaki moleküler taklitten kaynaklanır (1-3). RA'nın en ciddi belirtilerinden olan kardit, hastaların %30-45'inde ilk atak sırasında gelişebilse de, RKKH çoğunlukla tekrarlayan semptomatik akut RA epizodlarına bağlı kümülatif kapak hasarından kaynaklanmaktadır (4,5). Özellikle gelişmekte olan ülkelerde oldukça ciddi bir sağlık problemi haline gelen RKKH, çocuklarda ve genç yetişkinlerde kalp yetmezliğinin önde gelen nedeni olup, sakatlık ve/veya erken ölümle sonuçlanabilir. RKKH'da, hastalar kalp yetmezliği ile başvurana kadar tespit edilememektedir. Bu aşamadaki hastalar için tek tedavi seçeneği olan ameliyat ciddi komplikasyonlara sahiptir. Bu ciddi komplikasyonların önlenmesinde nüfusun RKKH için taranması ve duyarlı bireylerin belirlenmesi hastalığın erken teşhis, hedefe yönelik tedavi seçeneklerinin uygulanması için uygun biyobelirteçlerin belirlenmesi oldukça önem arz etmektedir. Diğer yandan, β hemolitik grup A streptokok enfeksiyonuna maruziyet sonrasında bireylerin yaklaşık olarak %3-6'sında RKKH gelişmektedir ve bu durum özellikle son zamanlarda yapılan çalışmalarda bireyler arasındaki genetik farklılıklara atfedilmektedir (6-8). Hastalığın patofizyolojisi henüz tam olarak aydınlatılamamış olsa da çok sayıda sitokin geni RKKH ile ilişkilendirilmiştir. Enflamasyona karşı konak yanıtının ürünleri olan sitokinler, enfeksiyonlara karşı savunmada önemli rol oynamaktadır. Enfeksiyonlar sırasında sitokinler, bağışıklık tepkisinin temel aracılarıdır ve bir dizi uyarıcı veya inhibe edici düzenleyici sinyaller açığa çıkararak çeşitli bağışıklık sistemi hücreleri üzerinde etki gösterirler. Sitokin genlerinde meydana gelen varyasyonların sitokin üretimini ve salgılanmasını etkileyerek bireyin hastalığa olan yatkınlığını, hastalığın şiddetini ve hatta tedaviye yanıtını da etkilediği bilinmektedir. RA/RKKH hastalarında *TNF-α, IL-1, IL-6, IFN-γ*'nın da dahil olduğu proenflamatuvar sitokinlerin plazma seviyelerinin yüksek olduğu bildirilmiştir (9-12). Bizim de 2018 yılında yapmış olduğumuz çalışma verileri *IFN-γ* varyantları ve RKKH arasındaki ilişkiyi belgeler niteliktedir (13). İnterlökin-1 (*IL-1*) gen kümesi, proenflamatuvar sitokin grubundan *IL-1α, IL-1β* ve bunların rekabetçi inhibitörü IL-1 reseptör antagonistini (*IL-1RA*) ifade eden genler kromozom 2 üzerinde yerleşmiştir (14). *IL-1Ra* geni intron 2'de değişken sayıda ardışık yinelemelere (VNTR) sahip bölgeler içermekte olup bu varyasyon hem *IL-1Ra* hem de *IL-1β* üretiminde kantitatif farklılıklara neden olabilmektedir (15,16). *IL-1Ra* A2 alleli, uzun süreli ve daha şiddetli bir proenflamatuar bağışıklık tepkisi ile ilişkilendirilmiştir. Çeşitli çalışmalarda, *IL-1Ra* A2 homozigotların RKKH dahil olduğu otoimmün ve kronik enflamatuvar durumlarla bağlantılı olabileceği bildirilmiştir (17,18). Ancak, RKKH patogenezinde *IL-1Ra* varyantının ilişkisi ile ilgili çalışmalar oldukça sınırlıdır. Biz çalışmamızda, Türk toplumunda *IL-1Ra* varyantları ile RKKH duyarlılığı ve/veya hastalığın şiddeti arasında bir ilişki olup olmadığını belirlemeyi amaçladık.

GEREÇ VE YÖNTEM

Çalışma populasyonumuz, Giresun Üniversitesi Tıp Fakültesi kardiyoloji polikliniği tarafından takip edilen baskın mitral darlığı ve hafif-orta derecede mitral yetersizliği ve eşlik eden darlıklı hafif-orta ikinci veya üçüncü kapak hastalığı olan 165 kadın RKKH hastasından (yaş: 50,14±13,99) ve ekokardiyografide kalp kapak hastalığı tespit edilmeyen, otoimmün hastalığı olmayan, ailede RKKH öyküsü olmayan sağlıklı (yaş: 49,72±12,80) 300 kadın kontrolden oluşmaktadır. Çalışmaya dahil edilmeden önce her hasta ve kontrolden bilgilendirilmiş onam alınmıştır. Tüm hastalar transtorasik ekokardiyografi ile 2015 Jones kriterlerine göre RKKH tanısı açısından tekrar değerlendirilmiştir (19). Kapak hasarının ciddiyeti, mitral balon valvotomi (MBV) endikasyonları ve mitral kapak replasmanı (MVR), ACC/AHA/ASE 2003 Ekokardiyografinin Klinik Uygulaması İçin Kılavuz Güncellemesi 2008'e göre değerlendirilmiştir. Kapak lezyonları, ekokardiyografi ile teşhis edilen romatizmal kalp kapak hastalığı, mitral darlık ve/veya yetersizlik ve eşlik eden hafif-orta dereceli ikinci veya üçüncü kapak hastalığı ile ilişkilidir. Çalışmamızda, dejeneratif aort darlığı olan hastalar göz ardı edilmemiştir. Şiddetli kapak hasarı olan MBV ve/veya MVR olan hastaların klinik ve ekokardiyografik verileri geriye dönük olarak ekokardiyografik verilerden değerlendirilmiştir. Fonksiyonel kapasite New York Kalp Cemiyeti'nin (*NYHA*) sınıf II – III semptomları, istirahatte pulmoner arter sistolik basıncı >50 mm Hg, mitral kapak alanı <1,5 cm^2, hiç olmayan veya hafif-orta mitral yetersizliği olan hastalar ve sol atriyal pıhtısı olmayan ve MBV için uygun kapak morfolojisinin uygun olduğuna karar verilmiştir. Fonksiyonel kapasitesi NYHA sınıf III – IV, mitral kapak alanı <1,5 cm^2 ve pulmoner arter sistolik basıncı >50 mm Hg olan ve orta ila şiddetli mitral yetersizliği olan hastalar MVR gerekliliğinin göstergesi olarak alınmıştır. Hastalar şiddetli kapak hastalığı (SKH), (n:122, %73,9; yaş: 48±13) veya hafif kapak hastalığı (HKH), (n:43, %26,06; yaş: 49±13) olarak kategorize edilmiştir. SKH grubu, MBV ve/veya MVR öyküsü olan hastalardan veya SKH'lı hastalardan ve MVR adaylarından oluşmaktadır. Hastaların 30'unda başlangıçta MVR, 60'ında (%36,36) MBV, 26'sında (%15,15) daha sonra MVR gelişti ve 54'ü (%32,72) MVR için adaydı**.** HKH grubu, fiziksel muayeneler ve ekokardiyografik veriler yoluyla hafif-orta şiddetli kapak hastalığı belirtileri nedeniyle tıbbi takipte olan asemptomatik hastalardan oluşuyordu. Ekokardiyografik bulgulara göre; hastalık başvurusundaki hastaların temel özellikleri Tablo 1'de sunulmuştur. Bu çalışmadaki tüm işlemler, kurumsal ve/veya Ulusal Araştırma Komitesinin Etik Standartlarına ve Helsinki Bildirgesine uyumlu olarak gerçekleştirilmiştir (20). Çalışmamız Giresun Üniversitesi Tıp Fakültesi Klinik Araştırmalar Etik Kurulu'ndan alınan (Etik Kurul No: KAEK-121) etik kurul kararınca gerçekleştirilmiştir.

IL-1Ra Genotip Analizi

Çalışmaya dahil edilen olgulardan alınan periferik kandan ticari kit (Roche high pure isolation kit, Germany) ile DNA izole edilmiş, saflık tayini yapılmış, ve DNA düzeyi hesaplandıktan sonra çalışma zamanına kadar +4^{0}C'de saklanmıştır. *IL-1Ra* gen bölgesinin amplifikasyonu için klasik polimeraz zincir reaksiyonu (PZR) yöntemi ve F: 5´-CTCAGCAACACTCCTAT-3´ R: 5´-TCCTGGTCTGCAGGTAA-3´ oligonükleotidleri kullanılmıştır. 86 bp'lik

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Tablo 1. Çalışma gruplarına ait karakteristik bilgiler.		
Parametreler	**Yaş (yıl)**	
Kontrol (n=300)	49,72±12,80	p=0,757
Hasta (n=165)	50,14±13,39	
Hasta sınıflandırması		**n (%)**
Mitral kapak lezyonları		
HKH	49±13	43 (%26,06)
SKH	48±13	122 (%73,9)
Mitral kapak		128 (%77,5)
Mitral Kapak + Aort Kapak		37 (%22,4)
Kapak replasmanı (KR)		
KR+		65 (%39,3)
KR-		100 (%60,6)
Valvuloplasti (VP)		
VP+		87 (%52,7)
VP-		78 (%47,2)

Ortalama değerler, Student's t-testi kullanılarak hastalar ve kontroller arasında karşılaştırılmıştır. Niteliksel veriler ki-kare testi ile analiz edilmiştir. Veriler ortalama±S.D ve n (%) olarak sunulmuştur. HKH: Hafif kapak hasarı. SKH: Şiddetli kapak hasarı.

VNTR içeren *IL-1Ra* geninin intron 2'si içindeki polimorfik bölge için PZR protokolü; 95^{0}C'de 5 dakika başlangıç denatürasyonunu takiben; 35 döngü halinde 95^{0}C'de 45 saniye denatürasyon, 55^{0}C'de 45 saniye bağlanma, 72^{0}C'de 45 saniye uzama aşamaları ve takibinde son olarak 72^{0}C'de 10 dakika olarak gerçekleştirilmiştir. Elde edilen PZR ürünleri %2'lik agaroz jelde UV altında görüntülenerek genotiplendirme yapılmış ve tekrar sayısına göre alleller karakterize edilmiştir (17). A1 allel (dört tekrar) 410 bp, A2 allel (iki tekrar) 240 bp, A3 allel (beş tekrar) 500 bp'dan oluşmaktadır.

İstatistiksel Analiz

Bu çalışmanın istatistiksel analizi SPSS 20 paket programı kullanılarak yapılmıştır. Tüm allel ve genotip frekansları doğrudan sayma ile hesaplanmıştır. Hardy-Weinberg dengesi (HWE), Arlequin V3.0 yazılımı kullanılarak hesaplanmıştır (21). Genotip ve allellerin görülme sıklığının gruplar arası farklılıklarının değerlendirilmesinde ki kare (χ^2) testi kullanılmıştır. Gruplar arası risk etkeninin belirlenmesi için odds oranı (OR) ve %95 güven aralığı (%95 GA) verilmiştir. İstatistiksel anlamlılık sınırı p<0,05 olarak alınmıştır.

BULGULAR

Çalışmamıza 465 kadın (165 hasta + 300 kontrol) birey dahil edilmiştir. Hasta (yaş: 50,14±13,39) ve kontrol (yaş: 49,72±12,80) grupları arasında yaş açısından istatistiksel olarak anlamlı bir fark bulunmamaktadır. RKKH hastalarında ve kontrollerde *IL-Ra* VNTR genotip ve allel frekansları Tablo 2'de gösterilmektedir. Hasta grubunda Hardy-Weinberg dengesinden sapma gözlenmiştir (χ2=4,32; p=0,645). Bununla birlikte, A1/A1 genotipi RKKH'ya yatkınlık ile ilişkili olduğundan, hasta populasyonunda homozigotluk artışının HWE'den sapmaya neden olan seçimden kaynaklanıyor olabileceği varsayılabilir. Çalışmaya dahil edilen hasta ve

Tablo 2. Hasta ve kontrol gruplarına ait genotip ve allel dağılımları.								
Genotip								
	A1/A1		**A1/A2**		**A1/A3**	**A2/A2**	**A3/A3**	
Hasta (n=165)	71 (%43,0)		79 (%47,9)		10 (%6,1)	3 (%1,8)	2 (%1,2)	
Kontrol (n=300)	155 (%51,7)		104 (%34,7)		19 (%3,0)	32 (%10,7)	0 (%0,0)	
P değeri	**< 0,001**							
Allel frekansları								
	A1 (+)	**A1 (-)**	**A2 (+)**	**A2 (-)**	**A3 (+)**	**A3 (-)**	**A4 (+)**	**A5 (+)**
Genel (n=465)	0,56	0,44	0,29	0,71	0,03	0,97	0,0	0,0
Hasta (n=165)	0,97	0,03	0,49	0,50	0,073	0,92	0,0	0,0
Kontrol (n=300)	0,89	0,10	0,45	0,54	0,03	0,97	0,0	0,0
X^2	8,7476		0,814		4,507			
P Değeri	**0,004**		0,367		**0,034**			

Veriler n (%) olarak sunulmuştur. **Kalın** değerler istatistiksel olarak anlamlıdır (p <0.05). n: örnek sayısı. Tüm alleller allel frekansı şeklinde sunulmuştur.

kontrol gruplarında 86-bp'lik tekrar sayısının farklılıklarına göre yalnızca üç IL1-Ra VNTR alleli (A1,A2,A3) gözlenmiştir. Türk toplumunda bu allellerin genel populasyondaki frekans dağılımları diğer populasyonlara benzerlik göstermektedir. A1 (%56,9) en yaygın alleldir, ardından A2 (%29,9) ve A3 (%3,2) nadir allel olarak karşımıza çıkmaktadır. Hastalar ve kontroller arasında genotip ve allel frekanslarında istatistiksel olarak anlamlı fark olduğu gözlenmiştir (p<0,001). A1 alleli, RKKH hastalarında kontrol grubuyla karşılaştırıldığında, anlamlı olarak daha yüksek bulunmuştur (p=0,004; OR=3,821; %95 GA=1,459-10,005). A2 alleli hasta ve kontrol grubu arasında önemli bir fark göstermezken (p=0,8367; OR=1,191; %95 GA=0,814-1,743) A3 alleli tüm hasta grubunda kontrol grubuna göre istatistiksel olarak anlamlı şekilde artış göstermiştir (p=0,034; OR= 2,536; %95 GA=1,045-6,152).

Hasta grubumuzun tamamı kapak hasarı açısından incelendiğinde, şiddetli ve hafif kapak hasarı olan hastalar arasında genotip dağılımları açısından istatistiksel olarak anlamlı farklılık bulunmuştur (p=0,031*)*.

Hafif ve şiddetli kapak hasarı olan hastalarda *IL-Ra* VNTR alleli ve genotip frekansları Tablo 3'de gösterilmektedir. A2 alleli şiddetli kapak hasarı olan hastalarda hafif kapak hasarı olan hastalara kıyasla istatistiksel olarak anlamlı derecede farklıdır (p=0,003). A1 ve A3 alleli iki grup arasında istatistiksel olarak anlamlı fark göstermemiştir (sırasıyla, FE=1,000; p=0,050). *IL-Ra* VNTR alleli ve genotip frekansları kapak replasmanı ve valvüloplasti açısından incelendiğinde gruplar arasında fark bulunmamıştır (data gösterilmemiştir).

TARTIŞMA

IL-1 reseptör antagonisti, *IL1-α* ve *IL1-β* ile rekabet halinde *IL-1* reseptörlerine bağlanabilen, böylece aktivitelerini inhibe eden ve *IL-1* ile ilişkili çeşitli immün ve enflamatuvar aktiviteleri modüle edebilen önemli bir anti-enflamatuvar sitokindir (22,23). *IL-1R*a geninde (*IL1-RN*), intron 2'de üç potansiyel protein bağlanma bölgesi içeren 86 baz çifti tekrarı olan bir VNTR bulunmaktadır. Bu bağlanma bölgesi potansiyel transkripsiyon faktörü bağlanma bölgesinde bulunur ve bu nedenle bu bölgedeki polimorfizmlerin fonksiyonel sonuçlara sahip olması beklenir (24,25). Çalışmalar, RA/RKKH'ya yatkınlığın büyük ölçüde genetik faktörler tarafından belirlendiğini göstermiştir. Bu nedenle, RKKH' ya yatkınlıkla ilişkili konakçı genetik belirteçlerin belirlenmesi, duyarlı bireylerin saptanması için yararlı olabilir. Diğer yandan RKKH ve *IL-1Ra* varyasyonu ile ilişkili yapılan çalışmalar hem çok sınırlıdır, hem de sonuçları çelişkilidir. Bu nedenle, çalışmamızda Türk toplumunda bu varyasyonun RKKH ile ilişkisini belirlemeyi amaçladık. Çalışmamız, Türk populasyonu için RKKH'da *IL1-Ra* polimorfizminin etkisini gösteren ilk çalışmadır. Hasta ve kontrol grupları arasında genotip dağılımları açısından anlamlı fark bulunmuştur (p< 0,001). Settin ve arkadaşları, 50 Mısırlı çocukta yapmış oldukları çalışmada A1/A1 genotipi ve mitral kapak hastalığı arasında pozitif bir ilişki olduğunu göstermişlerdir (17). Bizim çalışmamızda da bu çalışmayla uyumlu olarak A1 allelinde hasta grubu kontrol grubu ile kıyaslandığında istatistiksel olarak artış tespit edilmiştir (p= 0,003). Bu bulgular, Azevedo ve arkadaşları tarafından 84 Brezilyalı RA hastasında A1 taşıma oranı ile A1/A1 genotipi arasında negatif bir ilişkinin bildirildiği çalışma verileri ile çelişmektedir (26). Bu durum, bir genin işlevinin genomik bağlamına bağlı olduğu ve aynı genin farklı ırklarda farklı ifade modellerine sahip olabileceği şeklinde açıklanabilir. İkinci bir adım olarak hasta grubumuzu şiddetli ve hafif kapak hasarı olan hastalar şeklinde ayırdığımızda uzun süreli enflamasyonla ilişkili olduğu bilinen A2 allelinin varlığı şiddetli kapak hasarı olan hastalarda istatistiksel olarak yüksek bulunmuştur (p= 0,003). Bulgularımız, Rehman ve arkadaşları-

Tablo 3. Şiddetli ve hafif kapak hasarı olan hastalarda genotip ve allel dağılımları.

Kapak hasarı								
Genotip	**A1/A1**		**A1/A2**		**A1/A3**	**A2/A2**		**A3/A3**
SKH (n=122)	47 (%38,5)		66 (%54,1)		5 (%4,1)	3 (%2,5)		1 (%0,8)
HKH (n=43)	24 (%55,8)		13 (%30,2)		5 (%11,6)	0 (%0,0)		1 (%2,3)
P değeri					***0,031***			
Allel fraksiyonu								
	A1 (+)	**A1 (-)**	**A2 (+)**	**A2 (-)**	**A3 (+)**	**A3 (-)**	**A4 (+)**	**A5 (+)**
SKH (n=122)	0,96	0,03	0,56	0,43	0,049	0,95	0,0	0,0
HKH (n=43)	0,97	0,02	0,30	0,69	0,14	0,86	0,0	0,0
X^2	0,098		8,814		3,849			
P değeri	FE=1,000		**0,003**		0,050			

Kalın değerler istatistiksel olarak anlamlıdır (p <0.05). Niteliksel veriler ki-kare testi ile analiz edilmiştir. n: örnek sayısı. Veriler n (%) olarak sunulmuştur. Tüm alleller allel fraksiyonu şeklinde sunulmuştur. HKH: Hafif kapak hasarı. SKH: Şiddetli kapak hasarı.

nın *IL-1Ra* A2 allelini homozigot taşıyanlarda, RKKH ile ilişkisini bildiren bulguları ile benzerlik göstermektedir (27). Diğer yandan, Çin kökenli Tayvanlı hastalarda *IL-1Ra* VNTR'nin hastalığın şiddeti ile ilişkisinin olmadığı sonucunu bulan Chou ve arkadaşlarının sonuçları ile çelişmektedir (28).

Bulgularımıza göre, A1 alleli RKKH'a yatkınlıkla ilişkili iken A2 allelinin kapak hasarının şiddeti açısından önemli olduğunu düşündürmektedir. Ancak, bu çalışmada ele alınması gereken bazı sınırlamalar vardır. Çalışmamız; 2019-2020 yılları arasında Giresun Üniversitesi kardiyoloji polikliniğine başvuran RKKH hastalarından oluşmaktadır. Bu dönem içerisinde kliniğe başvuru yapan hasta populasyonu kadın ağırlıklıdır. Bu nedenle istatistiksel güce ulaşmayan erkek hasta populasyonu çalışmadan çıkarılmıştır. Bu nedenle cinsiyet ve *IL-1Ra* VNTR arasındaki ilişkiyi belirleyemedik. İkinci sınırlılığımız, vücut kitle indeksi, enflamasyonla ilişkili biyokimyasal belirteç düzeyleri (CRP, ASO gibi) bulunmadığı için karşılaştırma ve korelasyon yapılamamıştır. Ek olarak, serum *IL-1Ra* seviyeleri ölçülememiştir. Çalışmamızdaki diğer sınırlılığımız, nispeten küçük olan örneklem büyüklüğümüzdür. *IL-1Ra* VNTR polimorfizminin RKKH gelişimi üzerinde etkisi olup olmadığını belirlemek için çalışmanın daha büyük örneklem grubunda (hasta ve kontrol grupları) yürütülmesi gerekmektedir. Bu nedenle, daha güvenilir sonuçlar elde etmek için *IL-1Ra* ekspresyon seviyeleri ve RKKH riski üzerindeki etkileri dahil olmak üzere daha büyük örneklem büyüklüğünde daha fazla çalışmaya ihtiyaç vardır.

SONUÇ

Sonuç olarak çalışmamız, *IL-1Ra* VNTR'nin RKKH'nın patogenezine katkısının olduğunu doğrulamaktadır. Bireyleri RKKH'a duyarlı hale getiren *IL-1Ra* VNTR polimorfizmi, RKKH ile ilişkili morbidite ve mortaliteyi azaltmak için profilaktik müdahaleden fayda sağlayacak duyarlı bireylerin belirlenmesi için uygun bir biyobelirteç olarak önerilebilir. Ancak, bu konudaki mekanizmayı tam olarak açıklığa kavuşturmak için daha ileri çalışmalara ihtiyaç vardır. Bizim çalışmamız, ileride yapılacak çalışmalar için bir ön veri niteliği taşımaktadır.

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KAYNAKÇA/REFERENCES

1. Cunningham MW. Rheumatic fever, autoimmunity, and molecular mimicry: the streptococcal connection. Int Rev Immunol 2014; 33(4): 314-9. [CrossRef]
2. Guilherme L, Kalil J, Cunningham M. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. Autoimmunity 2006; 39(1): 31-9. [CrossRef]
3. Poomarimuthu M, Elango S, Solomon PR, Soundarapandian S, Mariakuttikan J. Lack of Association Between TNF-α, IFN-γ, IL-10 Gene Polymorphisms and Rheumatic Heart Disease in South Indian Population. Fetal and Pediatric Pathology 2018; 37: 309-8. [CrossRef]
4. Marijon E, Mirabel M, Celermajer DS, Jouven X. Rheumatic heart disease. Lancet 2012; 10: 953-4. [CrossRef]
5. Guilherme L, Kalil J. Rheumatic fever: from sore throat to autoimmune heart lesions. Int Arch Allergy Immunol 2004; 134: 56-64. [CrossRef]
6. Carapetis JR, Currie BJ, Mathews JD. Cumulative incidence of rheumatic fever in an endemic region: a guide to the susceptibility of the population? Epidemiol Infect 2000; 124: 239-44. [CrossRef]
7. Guilherme L, Ramasawmy R, Kalil J. Rheumatic fever and rheumatic heart disease: genetics and pathogenesis. Scand J Immunol 2007; 66: 199-207. [CrossRef]
8. Engel ME, Stander R, Vogel J, Adeyemo AA, Mayosi BM. Genetic susceptibility to acute rheumatic fever: a systematic review and meta-analysis of twin studies. PLoS One 2011; 6 (9): e25326. [CrossRef]
9. Toor D, Vohra H. Immune responsiveness during disease progression from acute rheumatic fever to chronic rheumatic heart disease. Microbes Infect 2012; 14: 1111-7. [CrossRef]
10. Guilherme L, Kalil J. Rheumatic fever and rheumatic heart disease: cellular mechanisms leading autoimmune reactivity and disease. J Clin Immunol 2010; 30: 17-23. [CrossRef]
11. Col-Araz N, Pehlivan S, Baspinar O, Oguzkan-Balci S, Sever T, Balat A. Role of cytokine gene (IFN-c, TNF-a, TGF-b1, IL-6, and IL-10) polymorphisms in pathogenesis of acute rheumatic fever in Turkish children. Eur J Pediatr 2012; 171: 1103-8. [CrossRef]
12. Van Deventer SJ. Cytokine and cytokine receptor polymorphisms in infectious disease. Intensive Care Med 2000; 26: 98-102. [CrossRef]

13. Teker E, Akadam-Teker AB, Ozturk O, Eronat AP, Yalin K, Golcuk SE, et al. Association Between the Interferon Gamma 874 T/A Polymorphism and the Severity of Valvular Damage in Patients with Rheumatic Heart Disease. Biochem Genet 2018; 56: 225-34. [CrossRef]
14. Hirsch E, Irikura VM, Paul SM, Hirsh D. Functions of interleukin 1 receptor antagonist in gene knockout and overproducing mice. Proc Natl Acad Sci USA 1996; 93 (20): 11008-13. [CrossRef]
15. Tarlow JK, Blakemore AI, Lennard A, Solari R, Hughes HN, Steinkasserer A, et al. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genet 1993; 91(4): 403-4. [CrossRef]
16. Vamvakopoulos JE, Taylor CJ, Morris-Stiff CJ, Green C, Metcalfe S. The interleukin-1 receptor antagonist gene: a single-copy variant of the intron 2 variable number tandem repeat (VNTR) polymorphism. Eur J Immunogenet 2002; 29(4): 337-40. [CrossRef]
17. Settin A, El-Baz HA, Saber I. Gene polymorphisms of TNF-α-308, IL-10–1082, IL-6-174 and IL-RaVNTR related to susceptibility and severity of rheumatic heart disease. Pediatr Cardiol 2007; 28: 363-71. [CrossRef]
18. Witkin SS, Gerber S, Ledger WJ. Influence of Interleukin-1 receptor antagonist gene polymorphism on disease. Clin Infect Dis 2002; 34: 204-9. [CrossRef]
19. Gewitz MH, Baltimore RS, Tani LY, Sable C A, Shulman ST, Carapetis J, et al. Revision of the Jones Criteria for the Diagnosis of Acute Rheumatic Fever in the Era of Doppler Echocardiography: A Scientific Statement From the American Heart Association. Circulation 2015; 131: 1806-18. [CrossRef]
20. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 2013; 310(20): 2191-4. [CrossRef]
21. Excoffier L, Laval G, Schneider S. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 2005; 1: 47-50. [CrossRef]
22. Patterson D, Jones C, Hart I, Bleskan J, Berger R, Geyer D, et al. The human interleukin-1 receptor antagonist (IL1RN) gene is located in the chromosome 2q14 region. Genomics 1993; 15: 173-6. [CrossRef]
23. Carapetis J, Beaton A, Cunningham MW, Guilherme L, Karthikeyan G, Mayosi BM et al. Acute rheumatic fever and rheumatic heart disease. Nat Rev Dis Primers 2016; 2: 15084. [CrossRef]
24. Tarlow JK, Blakemore AI, Lennard A, Solari R, Hughes HN, Steinkasserer A et al. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. Hum Genetic 1993; 91(4): 403-4. [CrossRef]
25. Al-Tahhan M, Etewa RL, El Behery MM. Association between circulating interleukin-1 beta (IL-1β) levels and IL-1β C-511 T polymorphism with cervical cancer risk in Egyptian women. Mol Cell Biochem 2011; 353: 159-65. [CrossRef]
26. Azevedo PM, Bauer R, Caparbo F, Silva CA, Bonfa E, Pereira RM. Interleukin-1 receptor antagonist gene (IL1RN) polymorphism possibly associated to severity of rheumatic carditis in a Brazilian cohort. Cytokine 2010; 49: 109-13. [CrossRef]
27. Rehman S, Akhtar N, Saba N, Munir S, Ahmed W, Mohyuddin A, et al. A study on the association of TNF-a-308, IL-6-174, IL-10-1082 and IL-1RaVNTR gene polymorphisms with rheumatic heart disease in Pakistani patients. Cytokine 2013; 61: 527-31. [CrossRef]
28. Chou HT, Tsai CH, Chen WC, Tsai FJ. Lack of association of genetic polymorphisms in the interleukin-1beta, interleukin-1 receptor antagonist,interleukin-4, and interleukin-10 genes with risk of rheumatic heart disease in Taiwan Chinese. Int Heart J 2005; 46: 397-406. [CrossRef]

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Antimicrobial Activity of *Pulicaria* Species from Turkey

Türkiye'deki *Pulicaria* Türlerinin Antimikrobiyal Aktivitesi

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ABSTRACT

Objective: In traditional medicine, the *Pulicaria* species are used for various disorders such as inflamed wounds, skin diseases, and bronchitis. This study investigated the antibacterial effect of five *Pulicaria* species in Turkey; (*Pulicaria (P) arabica* (L.) Cass., *P. dysenterica* (L.) Bernh., *P. odora* (L.) Reichb., *P. sicula* (L.) Moris, *P. vulgaris* (L.) Gaertn.) against certain significant pathogenic gram-negative and gram-positive reference bacteria.

Material and Method: Four extracts (decoction, infusion, aqueous, and ethanol (EtOH) extracts) were prepared from the *Pulicaria* species. The antimicrobial activity of the samples was examined against reference organisms; *Bacillus (B) subtilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Methicillin-resistant Staphylococcus (S) aureus (MRSA), Proteus mirabilis, S. aureus* and *Pseudomonas aeruginosa*. The minimal bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) ranges of the extract samples were demonstrated based on a microbroth dilution method.

Results: The EtOH extracts of the studied four *Pulicaria* species were found to be weakly active against gram-positive bacteria such as *B. subtilis*, MRSA and *S. aureus* but the EtOH extract of *P. dysenterica* showed exceptionally good activity against the reference strains of *S. aureus*, MRSA. No antimicrobial activity was detected in the infusion, decoction, and aqueous extracts.

Conclusion: The *Pulicaria* species, especially *P. dysenterica* could be evaluated as antimicrobial agents. Further studies with the extracts and essential oils from *Pulicaria* sp. on other bacteria and pathogenic fungi should be performed.

Keywords: *Pulicaria*, antimicrobial activity, extracts, Turkey

ÖZ

Amaç: Geleneksel tıpta *Pulicaria* türleri iltihaplı yaralar, cilt hastalıkları, bronşit gibi çeşitli rahatsızlıklarda kullanılmaktadır. Bu çalışmada Türkiye'deki beş *Pulicaria* türünün (*Pulicaria (P) arabica* (L.) Cass., *P. dysenterica* (L.) Bernh., *P. odora* (L.) Reichb., *P. sicula* (L.) Moris, *P. vulgaris* (L.) Gaertn.), bazı önemli patojenik gram-negatif ve gram-pozitif referans bakterilere karşı etkisi araştırılmıştır.

Gereç ve Yöntem: *Pulicaria* türlerinden dört ekstre (kaynatma, infüzyon, sulu ve etanol (EtOH) ekstreleri) hazırlandı. Örneklerin, antimikrobiyal aktivitesi referans organizmalara karşı incelendi; *Bacillus (B) subtilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae,* Metisiline dirençli *Staphylococcus (S) aureus* (MRSA), *Proteus mirabilis, S. aureus* ve *Pseudomonas aeruginosa*. Ekstre numunelerinin minimum bakterisidal konsantrasyon (MBC) ve minimum inhibitör konsantrasyon (MIC) aralıkları, mikrobroth seyreltme yöntemi temel alınarak gösterilmiştir.

Bulgular: İncelenen dört *Pulicaria* türünün EtOH ekstrelerinin; *B. subtilis,* MRSA ve *S. aureus* gibi gram pozitif bakterilere karşı genellikle zayıf aktif olduğu bulunmuştur, ancak *P. dysenterica*'nın EtOH ekstresi, *S. aureus*, MRSA referans suşlarına karşı çok iyi aktivite göstermiştir. İnfüzyon, dekoksiyon ve sulu ekstrelerde antimikrobiyal aktivite tespit edilememiştir.

Sonuç: *Pulicaria* türleri, özellikle *P. dysenterica* antimikrobiyal ajan olarak değerlendirilebilir. *Pulicaria* sp.'den elde edilen ekstreler ve uçucu yağlar ile diğer bakteri ve bazı patojenik mantarlar üzerinde daha ileri çalışmalar yapılmalıdır.

Anahtar Kelimeler: *Pulicaria*, antimikrobiyal aktivite, ekstreler, Türkiye

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INTRODUCTION

The genus *Pulicaria* Gaertn. is represented with about 149 taxa in the world (1). The *Pulicaria* species are widely used in traditional Turkish medicine. For instance: the species are used for bronchitis, colds, and inflamed wounds in Turkey (2), for diabetes, ankle sprains, headaches, and flatulent colic in Yemen (3), as cicatrizant, anti-inflammatory, for muscular-skeletal diseases, and skin diseases in Spain (4,5), and for abdominal pain in Mauritania (6). These species are also used in folk veterinary medicine in Italy as antiparasitics, repellents, and for respiratory ailments (7).

Distinct types of *Pulicaria* phytochemicals have been reported, including essential oils, flavonoids, phenolic derivatives, monoterpene derivatives, sesquiterpenes, diterpenes, triterpenes, steroids, and others (8). Mohamed et al. (2020) demonstrated that methanolic crude extracts of *Pulicaria (P) undulata and P. crispa* were rich in coumarins, saponins, sterols, tannins, and terpenes based on preliminary phytochemical screening (9). Until that time no flavonoids, alkaloids, and anthraquinones were found. According to identification of the essential oils of *P. crispa*; 1.4-ditert-butylbenzene, carvone, caryophyllene, and neryl (s)- 2-methylbutanoate were determined as the main compounds. The percentages of active ingredients are given respectively; 22.81%, 11.80%, 13.19%, and 10.33%. In the same study the main compounds of *P. undulata* essential oil were determined as camphor, thymyl acetate, bicycle, and azulenol. The percentages of active ingredients are given respectively 44.48%, 10.31%, 3.46%, and 3.40%. In the literature, there are several studies on essential oil constituents of *Pulicaria* species (10-16).

In the literature, there are several biological activity studies on *Pulicaria* species such as antimicrobial, antioxidant, anticholinesterase, analgesic, antipyretic, anti-inflammatory, cytotoxicity (HL-60, MCF-7, Hep-G2 cells), and hepatoprotective (11,14,17-22). The number of studies on the antimicrobial activities of the medicinal plant as a potential antimicrobial drug which functions as new antibacterial agents have increased in recent years (23). The antimicrobial activity of essential oils, plant extracts or isolated compounds used in traditional medicines is the subject of several studies. This study was conducted with the idea of determining the antimicrobial activities of *Pulicaria* species to illuminate the traditional uses of wound healing, bronchitis, colds, skin diseases, and abdominal pain.

The main goals of this research were to find out how effective five *Pulicaria* species (*P. arabica* (L.) Cass., *P. dysenterica* (L.) Bernh., *P. odora* (L.) Reichb., *P. sicula* (L.) Moris, *P. vulgaris* (L.) Gaertn.) were at fighting bacteria. Aqueous extracts, ethanol (EtOH) extracts, infusion, and decoction of these five *Pulicaria* species were evaluated against the reference strains of forementioned microorganisms.

MATERIAL AND METHOD

Plant Materials

Pulicaria species were gathered between 2018-2019. The studied species, herbarium numbers, and localities are given in Table 1. The plants were identified by Dr. Bahar Gürdal and Dr. Ebru Özdemir Nath. The *Pulicaria* species are stored in Istanbul University Herbarium of the Faculty of Pharmacy (ISTE).

Extraction of Plant Materials

For each species, four extracts (infusion, decoction, aqueous, and EtOH extracts) were prepared from aerial parts. The diverse techniques of extract preparation were chosen to compare the biological activity of traditionally made decoctions and infusions to water and EtOH extracts prepared under controlled laboratory circumstances. At room temperature, the plant materials were air-dried and powdered. For EtOH extracts, 50 grams (g) of each species were macerated with EtOH (250 mL, Merck) for 24 hours at room temperature and filtered. The solvents were evaporated to dryness under vacuum (Buchi Rotavapor R-210). For aqueous extracts, 5 g of each species were macerated with distilled water (25 mL) for 24 hours. Then it was filtered. Infusions were prepared by adding 25 mL of boiling distilled water to each species (5 g) and allowed to stand at room temperature for 20 min. For decoction, 5 g of each species were added to 25 mL of distilled water and heated for 20 minutes, and then filtered.

Determination of Antimicrobial Activity

The antibacterial effect of the plant extracts was evaluated against some of the reference bacteria using a standard microbroth dilution technique as defined in the guideline of Clinical Laboratory Standards Institute (24). The test organisms were provided from the Microorganism Culture Collection of Istan-

Table 1. Studied species information.

Species	Locality	Herbarium number
P. arabica	Muğla: Bodrum, Göltürkbükü, 19.vii.2019, B. Gürdal, E. Özdemir Nath	ISTE 116891
P. dysenterica	İstanbul: Çatalca, Subaşı, 14.viii.2018, B. Gürdal, E. Özdemir Nath	ISTE 116730
P. odora	Yalova: Armutlu, 06.vi.2019, B. Gürdal, E. Abamor	ISTE 116747
P. sicula	Bursa: Nilüfer, Gölyazı, 19.x.2019, B. Gürdal, E. Özdemir Nath	ISTE 116910
P. vulgaris	Edirne: Enez, Sultaniçe, 20.viii.2019, B. Gürdal, E. Özdemir Nath	ISTE 116895

bul University, Department of Pharmaceutical Microbiology. The reference antimicrobials, Ciprofloxacin (Bayer Türk Kimya San. Istanbul/Turkey) as antibiotic and dimethyl sulfoxide (Merck, Darmstadt, Germany) as a solvent and growth mediums Mueller Hinton Broth (MHB) and tryptic soy agar (TSA) were obtained from BD Difco (Fisher Scientific, Göteborg, Sweden).

DMSO was used as a solvent for EtOH extracts with a concentration of 10,000µg/mL and as a vehicle control to detect the possible inhibitory activity of the extract dilution. Ciprofloxacin was used as a reference antibiotic. The minimum inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) were determined for each extract.

Examination of MIC and MBC Assays

Inoculums were prepared from overnight cultures from each microorganism in MHB medium. The density was adjusted using McFarland densitometer (Biosan, Riga, Latvia) equivalent to 0.5 McFarland and diluted 1/100 to the final concentration 10^6 CFU/m.

After the preparation of inoculums, the microtiter plate wells were inoculated with 50 µg/mL MHB starting from the second until the last well. 10,000 µg/mL from EtOH extracts and 100,000 µg/mL concentration from infusion, decoction, and maceration extracts were inoculated to the first two wells. Two-fold serial dilutions were made (starting concentration of 10,000 µg/m L and 100,000 µg/mL in the first well to the minimum concentration 19.52 µg/mL and 195.2 µg/mL in the tenth well respectively). Finally, bacterial suspensions and 25 µl of resazurin solution (0.001%) were inoculated to each well, except the negative control well, and the range of 5000-9.76 µg/mL and 50.000-97.6 µg/mL were achieved, respectively.

The standard antibiotic ciprofloxacin's 2-fold serial dilutions were prepared in cation-adjusted MHB (CAMHB), in the ranges 5,120 to 64 mg/L. 50 µL from 32 to 0.03 µg/mL concentrations of the antibiotic solution as well as DMSO were directly inoculated to each corresponding microtiter well by serial dilutions and the microtiter plates were incubated at 37°C for 24 hours. Microbial growth in the wells was determined as positive which turned from lilac to pink by resazurin dye. The MIC was defined as the lowest concentration of the samples that completely inhibits the growth of microorganisms. To determine the MBC, broth samples were removed from the wells with no growth and placed on the TSA plates overnight at 37°C. MBCs were detected with samples that did not show any bacterial growth.

RESULTS

The EtOH extract of *P. sicula* showed activity against *Enterococcus (E) faecalis, Methicillin-resistant Staphylococcus (S) aureus (MRSA),* and *S. aureus* and the EtOH extracts of *P. arabica*, *P. vulgaris* and *P. odora* showed almost the same activity against *S. aureus*, MRSA, and *E. faecalis*. The EtOH extract of *P. odora* was also active against *Bacillus (B) subtilis*. Especially the EtOH extract of *P. dysenterica* was found active against most of the reference organisms. The MIC/ MBC results of the EtOH extract of *P. dysenterica* against MRSA, *S. aureus*, *E. faecalis*, *Proteus mirabilis* and *Klebsiella (K) pneumoniae* are 39/156 µg/mL, 156 µg/mL, 1250 µg/mL, 625/1250 µg/mL, and 625/1250 µg/mL, respectively as shown in Table 2.

The findings show that the EtOH extracts of *Pulicaria* species in Turkey are especially active against gram-positive pathogens such as *MRSA, S. aureus* and *E. faecalis* with a decrease up to 2-64 times in MIC values compared to DMSO, as shown in Table 2. In each case, the activities of the EtOH extracts of *Pulicaria* species were lower than those of the standard antibiotic ciprofloxacin. No antimicrobial activity was detected in the infusion, decoction, and aqueous extracts. It has been also determined that the *Pulicaria* extracts have not shown any antibacterial activity against some of the clinically important pathogenic bacteria such as *Pseudomonas aeruginosa* and *Escherichia (E) coli.*

Table 2. The results of antimicrobial assays (*Pulicaria* EtOH extracts, MICs/MBCs, µg/mL).

***Pulicaria* sp.**	***E. coli***	***S. aureus***	***MRSA***	***E. faecalis***	***K. pneumoniae***	***B. subtilis***	***Pseudomonas aeruginosa***	***Proteus mirabilis***
P. sicula	1250	625	312.5	2500	1250	1250	1250	1250
P. arabica	1250	625/1250	625	1250/2500	1250	1250	1250	1250
P. vulgaris	1250	1250	625	1250/2500	1250	1250	1250	1250
P. odora	1250	625/1250	625	1250/2500	1250	625/1250	1250	1250
P. dysenterica	1250	156	39/156	1250/1250	625/1250	1250	1250	625/1250
DMSO	1250	2500	2500	2500	1250	1250	1250	2500
Ciprofloxacin	0.125	1	0.5	0.5	0.5	0.5	0.5	0.5

Reference strains: Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, MRSA (methicillin-resistant S. aureus) ATCC 43300, Enterococcus faecalis ATCC 29212, Klebsiella pneumoniae ATCC 4352, Proteus mirabilis ATCC 14153, Pseudomonas aeruginosa ATCC 27853, Bacillus subtilis ATCC 6633

DISCUSSION

In the studies conducted to ascertain the antimicrobial activities of the *Pulicaria* species, successful results on gram-positive and gram-negative, clinically important bacteria were obtained in different extracts. Nickavar and Mojab, (25) assessed the methanolic, aqueous, and chloroform extracts of *P. dysenterica* aerial parts from Tehran, Iran for their antibacterial effect using the disc-diffusion technique. The six bacteria - *E. coli, Bacillus cereus, Shigella dysenteriae*, *Salmonella typhi*, *Vibrio cholera* and *S. aureus* - were tested and the most powerful extract against *V. cholera, B. cereus, S. aureus* and was the methanolic extract. All the extracts against *Vibrio cholera* were successfully active. Similar to this study, the EtOH extract of *P. dysenterica* has good activity against tested gram-positive bacteria. In both studies, there were not any activity against one of the significant pathogenic gram-negative bacteria such as *E. coli*.

Touati et al. (26), researched the antibacterial activity of leaves and roots of *P. odora* from northern Algeria. The findings showed that *S. aureus* was the most sensitive to the acetonic root extract, while *Pseudomonas aeruginosa* was the most resistant to the chloroformic leaf extract. According to Naqvi et al. (27), the findings of the analysis revealed that EtOH extract of *P. gnaphalode* from Quetta, Pakistan demonstrated a maximum inhibition zone for *B. subtilis* from all others. The methanol extract (ME) demonstrated a maximum inhibition zone for *S. aureus*. Zhanzhaxina et al. (28), analyzed the structure of the EtOH and $CHCl_3$ extracts of *P. vulgaris* from Akmola, Kazakhstan, and determined the biological function of the extracted compounds. All isolated compounds have been tested against *E. coli, S. aureus, B. cereus, Salmonella enteritidis,* and *Candida albicans*. The percent inhibition showed negative values in some cases, indicating that the compound did not decrease the number of bacteria. The antimicrobial activity of the CH_2Cl_2, MeOH, EtOAc extracts of the *P. undulata* aerial parts from Egypt was investigated by Abdel Bar et al. (29). The antimicrobial activity was measured against *S. aureus*, *K. pneumoniae*, *E. coli,* and *Pseudomonas aeruginosa*, and *C. albicans*. The phenolic rather than the terpenoidal compounds exhibited remarkable antimicrobial activity. The research of Foudah et al. (30), was intended to question the antimicrobial activity of the ME of *P. crispa* from Alkharj- the, Saudi Arabia. The antimicrobial activity was tested against *S.aureus, B. subtilis, E. coli, K. pneumonaie, Aspergillus niger*, *Proteus vulgaris,* and *C. albicans*. Antimicrobial activities were observed which may be due to the presence of phenols, tannins, and flavonoids in the ME. The study of El-Shahaby et al. (31) aimed to determine the antimicrobial potential of ethyl acetate and diethyl ether extracts of *P. incisa* (Lam.) DC from Egypt. The antimicrobial activities of extracts against *K. pneumoniae, B. subtilis, S. epidermidis, S. aureus, E. coli,* and *C. albicans* were determined by the disc diffusion technique. As a result of this study, the ethyl acetate extract has antimicrobial effect on *S. epidermidis, S. aureus* and *C. albicans* while the diethyl ether has activity against *B. subtillis* and *C. albicans.* Nair et al. (32) screened for antibacterial activity of *P. wightiana* from Rajkot Gujarat, India. Agar disc diffusion assay was used for aqueous extract and Agar disc diffusion assay used for ME against *Staphylococcus epidermidis, Pseudomonas testosteroni, B. subtilis, Proteus morganii, Micrococcus flavus,* and *K. pneumoniae.* The ME showed greater activity than the aqueous extract. The methanolic extracts are active against *Micrococcus flavus, B. subtilis* and *Proteus morganii.* The aqueous extract revealed a negligible amount of action. In this study, the aqueous extracts of studied *Pulicaria* species also showed the same results.

CONCLUSION

In this research, the antimicrobial activity of five species of *Pulicaria* in Turkey was investigated by the microdilution method modified with resazurin. The EtOH extracts obtained from *Pulicaria* species were found to be weakly active against the gram-positive bacteria such as *B. subtilis,* MRSA, *S. aureus*, but the EtOH extract of *P. dysenterica* showed particularly good activity against *S. aureus*, MRSA. These results suggest that the EtOH extract of *P. dysenterica* would be a good therapeutic agent against these bacteria. Further studies such as *in vitro* antimicrobial activity testing are suggested to evaluate antifungal and antibacterial activities of essential oils obtained from active *Pulicaria* species in Turkey.

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REFERENCES

1. The Plant List. 2010; Version 1. Available from: URL: http://www.theplantlist.org/
2. Tuzlacı E. Türkiye Bitkileri Geleneksel İlaç Rehberi. İstanbul: İstanbul Tıp Kitabevleri; 2016.
3. Al-Fatimi M. Ethnobotanical survey of medicinal plants in central Abyan governorate, Yemen. J Ethnopharmacol 2019; 241: 111973. [CrossRef]
4. Gonzalez-Tejero MR, Casares-Porcel M, Sanchez-Rojas CP, Ramiro-Gutierrez JM, Molero-Mesa J, Pieroni A, et al. Medicinal plants in the Mediterranean area: Synthesis of the results of the project Rubia. J Ethnopharmacol 2008; 116: 341-57. [CrossRef]
5. Vazquez FM, Suarez MA, Perez A. Medicinal plants used in the Barros Area, Badajoz Province (Spain). J Ethnopharmacol 1997; 55: 81-5. [CrossRef]
6. Yebouk C, Redouan FZ, Benítez G, Bouhbal M, Kadiri M, Boumediana AI, et al. Ethnobotanical study of medicinal plants in the Adrar Province, Mauritania. J Ethnopharmacol 2020; 246: 112217. [CrossRef]
7. Viegi L, Pieroni A, Guarrera PM, Vangelisti R. A review of plants used in folk veterinary medicine in Italy as basis for a databank. J Ethnopharmacol 2003; 89: 221-44. [CrossRef]
8. Liu L, Yang J, Shi Y. Phytochemicals and biological activities of Pulicaria species. Chem Biodivers 2010; 7(2): 327-49. [CrossRef]
9. Mohamed EAA, Muddathir AM, Osman MA. Antimicrobial activity, phytochemical screening of crude extracts, and essential oils constituents of two Pulicaria spp. growing in Sudan. Sci Rep 2020; 10(1): 1-8. [CrossRef]
10. Hanbali FE, Akssira M, Ezoubeiri A, Mellouki F, Benherraf A, Blazquez AM, et al. Chemical composition and antibacterial activity of essential oil of Pulicaria odora L. J Ethnopharmacol 2005; 99(3): 399-401. [CrossRef]
11. Ali NAA, Sharopov FS, Alhaj M, Hill GM, Porzel A, Arnold N, et al. Chemical composition and biological activity of essential oil from Pulicaria undulata from Yemen. Nat Prod Commun 2012; 7(2): 257-60. [CrossRef]
12. Fawzy GA, Al Ati HY, El Gamal AA. Chemical composition and biological evaluation of essential oils of Pulicaria jaubertii. Pharmacogn Mag 2013; 9(33): 28-32. [CrossRef]
13. Asghari G, Zahabi F, Eskandarian A, Yousefi H, Asghari M. Chemical composition and leishmanicidal activity of Pulicaria gnaphalodes essential oil. Res J Pharmacogn 2014; 1(4): 27-33.
14. Sharifi-Rad J, Miri A, Hoseini-Alfatemi SM, Sharifi-Rad M, Setzer WN, Hadjiakhoondi A. Chemical composition and biological activity of Pulicaria vulgaris essential oil from Iran. Nat Prod Commun 2014; 9(11): 1633-6. [CrossRef]
15. Chaib F, Allali H, Bennaceur M, Flamini G. Chemical composition and antimicrobial activity of essential oils from the aerial parts of Asteriscus graveolens (Forssk.) Less. and Pulicaria incisa (Lam.) DC.: Two Asteraceae herbs growing wild in the Hoggar. Chem Biodivers 2017; 14(8): e1700092. [CrossRef]
16. Zardi-Bergaoui A, Jelassi A, Daami-Remadi M, Harzallah-Skhiri F, Flamini G, Ascrizzi R, et al. Chemical composition and bioactivities of essential oils from Pulicaria vulgaris subsp. dentata (Sm.) Batt. growing in Tunisia. J Essent Oil Res 2020; 32(2): 111-20. [CrossRef]
17. Triana J, López M, Pérez FJ, González-Platas J, Quintana J, Estévez F, et al. Sesquiterpenoids from Pulicaria canariensis and their cytotoxic activities. J Nat Prod 2005; 68(4): 523-31. [CrossRef]
18. Yusufoglu HS. Analgesic, antipyretic, anti-inflammatory, hepatoprotective and nephritic effects of the aerial parts of Pulicaria arabica (Family: Compositae) on rats. Asian Pac J Trop Med 2014; 7: S583-S590. [CrossRef]
19. Hussien TA, El-Toumy S, Hassan H, Hetta M. Cytotoxic and antioxidant activities of secondary metabolites from Pulicaria undulata. Int J Pharm Pharm Sci 2016; 8(9): 150-5. [CrossRef]
20. Ravandeh M, Valizadeh J, Noroozifar M, Khorasani-Motlagh M. Screening of chemical composition of essential oil, mineral elements and antioxidant activity in Pulicaria undulata (L.) CA Mey from Iran. J Med Plant Res 2011; 5(10): 2035-40.
21. El-Kamali HH, Mahjoub SAT. Antibacterial activity of Francoeuria crispa, Pulicaria undulata, Ziziphus spina-christi and Cucurbita pepo against seven standard pathogenic bacteria. Ethnobot Leafl 2009; 13: 722-33.
22. Ali NAA, Crouch RA, Al-Fatimi MA, Arnold N, Teichert A, Setzer WN, et al. Chemical composition, antimicrobial, antiradical and anticholinesterase activity of the essential oil of Pulicaria stephanocarpa from Soqotra. Nat Prod Commun 2012; 7(1): 113-6. [CrossRef]
23. Rios JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol 2005; 100(1-2): 80-4. [CrossRef]
24. Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing: Approved Standard M100-S22. Wayne, Pennsylvania, 2012.
25. Nickavar B, Mojab F. Antibacterial activity of Pulicaria dysenterica extracts. Fitoterapia 2003; 74(4): 390-3. [CrossRef]
26. Touati N, Saidani K, Boudries H, Hammiche H, Ouazene N, Bedjou F. Antibacterial activity of phenolic compounds of Pulicaria odora, wild plant in northern Algeria. Int Food Res J 2018; 25(5): 2021-30.
27. Naqvi SAR, Shah SMA, Kanwal L, Saeed M, Atta-ul-Haq, Nisar J, et al. Antimicrobial and antihypercholesterolemic activities of Pulicaria gnaphalodes. Dose-Response 2020; DOI: 10.1177/1559325820904858. [CrossRef]
28. Zhanzhaxina AS, Seiilgazy M, Jalmakhanbetova RI, Ishmuratova MY, Seilkhanov TM, Oyama M, et al. Flavonoids from Pulicaria vulgaris and their antimicrobial activity. Chem Nat Compd 2020; 56(5): 915-7. [CrossRef]
29. Abdel Bar FM, Elsbaey M, Taha N, Elgaml A, Abdel-Fattah GM. Phytochemical, antimicrobial and antiquorum-sensing studies of Pulicaria undulata L.: A revision on the structure of 1β, 2α, 3β, 19α, 23-pentahydroxy-urs-12-en-28-oic acid. Nat Prod Res 2020; 34(6): 804-9. [CrossRef]
30. Foudah AI, Alam A, Soliman GA, Salkini MA, Ahmed EI, Yusufoglu HS. Pharmacognostical, antioxidant and antimicrobial studies of aerial part of Pulicaria crispa (Family: Asteraceae). Bull Environ Pharmacol Life Sci 2015; 4(12): 19-27. [CrossRef]
31. El-Shahaby O, El-Zayat M, Rabei R, Aldesuquy HS. Phytochemical constituents, antioxidant activity and antimicrobial potential of Pulicaria incisa (lam.) DC as a folk medicinal plant. Prog Chem Biochem Res 2019; 2(4): 222-7. [CrossRef]
32. Nair R, Kalariya T, Chanda S. Antibacterial activity of some selected Indian medicinal flora. Turk J Biol 2005; 29(1): 41-7.

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Do Norepinephrine and Estradiol Affect the Growth of *Escherichia coli* and Expressions of *Mar* Genes?

Norepinefrin ve Östradiol *Escherichia coli*'nin Üremesini ve Mar Genlerinin Ekspresyonunu Etkiler mi?

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ABSTRACT

Objective: Human hormones have been shown to regulate antibiotic resistance levels, pathogenicity, and growth of bacteria. In our study, we aimed to detect the possible effects of norepinephrine (NE) and estradiol (Est) on growth and expression of chromosomal multiple antibiotic resistance (mar) locus and related genes (*ompF, marA, tolC, acrA, marR* and *acrB*) in *Escherichia (E) coli* SPC105.

Material and Method: Serum supplemented SAPI (control) and serum SAPI containing norepinephrine (0.0017 µg/mL, 0.04 µg/mL, and 100 µg/mL) and estradiol (0.4 ng/mL, 3 ng/mL, 300 ng/mL) was used to grow *E. coli* SPC105. Growth alterations were determined using the turbidimetric method while the gene expression levels were examined by quantitative polymerase chain reaction (qPCR).

Results: It was shown that, NE and Est in all concentrations were shown to affect (reduce/enhance due to incubation periods or hormone concentrations) the growth of *E. coli* SPC105 apart from the high-level Est concentration. Expression levels of all six target genes were shown to be significantly enhanced in the presence of all concentrations of both NE and Est.

Conclusion: Our results constitute new data on the possible influences of these hormones on the growth and expressions of mar operon on transcriptional levels in the *E. coli* SPC105 strain.

Keywords: Norepinephrine, estradiol, mar operon, gene expression, growth, *E. coli*

ÖZ

Amaç: Hormonların bakterilerin virulansı, antibiyotik duyarlılığı ve üremesini düzenlediği bilinmektedir. Bu çalışmada, norepinefrin (NE) ve östradiolün (Est) *Escherichia (E) coli* SPC105 suşunun üremesi ve kromozomal çoğul antibiyotik dirençi (mar) lokusu ve ilişkili genlerin (*marA, marR, ompF, acrA, acrB* ve *tolC*) ekspresyonu üzerine olası etkileri incelenmiştir.

Gereç ve Yöntem: *E. coli* SPC105 suşu serum ilave edilmiş SAPI (kontrol olarak) ve norepinefrin (0,0017 µg/mL, 0,04 µg/mL ve 100 µg/mL) ile östradiol (0,4 ng/mL, 3 ng/mL, 300 ng/mL) eklenmiş serum-SAPI besiyerinde üretilmiştir. Üreme değişimleri turbidimetrik yöntem ile; gen ekspresyon düzeyleri ise kantitatif polimeraz zincir reaksiyonu (qPCR) ile araştırılmıştır.

Bulgular: Çalışmamızın sonucunda yüksek düzey Est dışında tüm denenen hormon konsantrasyonlarının *E. coli* SPC105 suşunun üremesi üzerine etkisi olduğu (inkübasyon süresine veya hormon konsantrasyonuna bağlı olarak baskılama/arttırma yönünde) gösterilmiştir. NE ve Est'nin tüm konsantrasyonlarının incelenen tüm hedef genlerin ekpresyonunu arttırdığı gösterilmiştir.

Sonuç: Sonuçlarımız incelenen bu iki hormonun *E. coli* SPC105 suşunun üremesi ve mar operonunun ekspresyonunda, transkripsiyonel seviyede, olası etkileri üzerine yeni bir veri ortaya koymuştur.

Anahtar Kelimeler: Norepinefrin, östradiol, mar operonu, gen ekspresyonu, üreme, *E. coli*

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INTRODUCTION

It is well known that not only host cells but also bacteria can sense and respond to human hormones as a result of a long coexistence and coevolution (1-3). Moreover, some microorganisms are also known to produce hormones which may be used to communicate with each other and to recognize their environment (quorum sensing pathway-QS) (1). It has been shown that human hormones may affect important biological processes such as growth, virulence, biofilm formations, antibiotic susceptibility, and expression levels of various genes of bacteria in a host during infection (1,4-6).

The emergence and spread of multiple antibiotic resistances in bacteria can occur through two different mechanisms: via the acquisition of genes or chromosomal mutations (7,8) and via intrinsic multidrug resistance (7) due to the regulation of chromosomal multiple antibiotic resistance (*mar*) locus. The conserved chromosomal *marRAB* operon in mar locus is found in *Escherichia (E) coli* and certain other enteric bacteria (*Citrobacter spp.*, *Klebsiella spp.*, *Shigella spp.*, *Enterobacter spp.*, *Hafnia spp.*, *Salmonella spp.*) (7-11).

The *marR*, *marA* and *marB* genes in *marRAB* operon encode MarR, MarA and MarB. Although the function of the MarB protein is not clearly understood, it is known that MarA and MarR are transcriptional regulatory proteins which are capable of binding to DNA and regulating the expression of *marRAB* operon (MarR is an auto repressor, MarA is an auto activator) (7,10). *marR* is inactivated by mutations or in the presence of certain phenolic ligands, antibiotics, and oxidative stress. If *marR* is inactive, *marRAB* becomes depressed and *marA* expression occurs. So, the organism becomes multiple antibiotic resistant (quinolones, tetracycline, β-lactams, organic solvents, oxidative stress agents and household disinfectants) due to the regulation of the efflux pump and outer membrane porin genes by MarA activations (8,11-13). Specifically, MarA is a global transcriptional activator and it has been shown to regulate nearly forty different genes including *acrA*, *acrB* and *tolC* encoding AcrAB-TolC multidrug resistance pump and *ompF* encoding outer membrane porin OmpF (10,14-16). Mar phenotypes were initially detected as being cross-resistant to quinolones, tetracycline, beta-lactams and various phenolic compounds (11). Bacteria become a mar phenotype when the AcrAB-TolC is over expressed and OmpF is down regulated (17).

In this study we aimed to detect the effects of norepinephrine (NE) (a stress hormone) and estradiol (Est) (a sex hormone) as host factors, on growth and expressions of the *acrA*, *acrB*, *marA*, *marR*, *omp*F and *tolC* genes associated with mar regulon in *E. coli* SPC105. These hormones are known to have specific phenolic groups like many other compounds such as various herbs, vegetables, and bile salts etc. which have also been shown to induce marRAB expression in *E. coli* SPC105 (17). The effects of these hormones on the expression of *marRAB* have not been investigated to date.

MATERIAL AND METHOD

Strain

The *E. coli* SPC105 strain carrying a chromosomal PmarII:lacZ fusion at the λ attachment site was used. This strain was kindly provided by Dr. Stuart Levy and Dr. Valérie Duval from Tufts University School of Medicine, USA. The organism was kept at -80°C for quantitative polymerase chain reaction (qPCR) analysis.

Medium, Hormones and Experimental Conditions

Previously defined, 30% (v/v) serum supplemented Standard American Petroleum Institute (serum-SAPI) was used (18). *E. coli* was inoculated in a hormone added SAPI medium to show the influences of hormones. Various levels of NE (0.0017 µg/mL, 0.04 µg/mL and 100 µg/mL) and Est (0.4 ng/mL, 3 ng/mL and 300 ng/mL) were analyzed. The mean blood levels of health conditions were used to determine the low and medium concentrations. In cases of different pathogenic circumstances to which bacteria may also be exposed, high levels were also selected (18, 19). SAPI with no hormone was used as a control medium. We kept the hormone concentrations at -20°C before the experiments and they were kept on ice during the experiments.

Detection of Alterations on Growth

E. coli SPC105 was inoculated in SAPI medium with no supplementation (as a control) and hormone added SAPI as mentioned above. The initial bacterial count ($4x10^6$ CFU/mL) was prepared in a four times dilution (1:3) of overnight culture. Bacteria were incubated at 37°C. Absorbance at 600 nm at the four, six- and 24-hour periods was measured to detect changes of growth. These conditions were tested in duplicate, and each experiment was performed twice.

Detection of Alterations on Gene Expressions

Gene expressions were determined according to transcriptional level of mRNAs in the presence and absence of host hormones (SAPI used as control).

Total RNA Isolation and cDNA Synthesis

E. coli SPC105 was grown in SAPI (control), SAPI+ 0.0017 µg/mL NE, SAPI+0.04 µg/mL NE, SAPI+100 µg/mL NE, SAPI+0.4 ng/mL Est, SAPI+3 ng/mL Est and SAPI+300 ng/mL Est for 16-24 hours at 37°C while shaking at 200 rpm. 1.5 mL of bacterial cultures was used in total RNA extraction using 0.5 mL TriPure reagent (Roche, Swiss). Total RNA extraction was carried out according to the manufacturer's recommendations. 0.5 ml Tri-Pure was added to the mortar and homogenization was completed. Homogenized liquid samples were transferred to micro tubes. After the transfer, 100 µL chloroform was added to each sample and shaken for 15 seconds to remove nucleoprotein complex. The samples were centrifuged for 15 minutes at 13,000 rpm and the colorless upper phase was transferred to new micro tubes. 250 µL isopropanol was added to the colorless phase and incubated at room temperature for 10 minutes. After the incubation, samples were centrifuged at 13,000 rpm for 10

minutes. Total RNA molecules precipitated to the bottom of the micro tube. The precipitated RNA molecules were solved with DEPC treated water. The isolated total RNAs were treated with 1 µL DNaseI (1mg/mL; Qiagen, Germany) at 37°C for 10 min. The mixture was treated with 1µL EDTA (50 mM) at 65°C for 5 min to stop any reaction. Quantitative and qualitative analysis of RNAs were performed with 1% agarose gel electrophoresis and spectrophotometer measurement (Thermo, USA), respectively. 2 µg total RNA molecules were converted with a cDNA commercial kit (Takara, Japan) according to the manufacturer's protocol. qPCR analysis was performed with ¼ diluted cDNA.

Quantitative Polymerase Chain Reaction Analysis

Eva Green (Bio-RAD, France) dye binding to double strand DNA (500ex/530em nm) was used in qPCR analysis. 16s rRNA gene was the selected housekeeping gene. Target genes' (marA, marR, ompF, acrA, acrB and tolC) expressions were normalized by expression of housekeeping gene. The $2^{-\Delta\Delta CT}$ formula developed by Livak and Schmittgen (20) was used to evaluate the expression levels. All analyses were carried out at least three times. Cp values were recorded and calculated using Quant Studio 5.0 software (Applied Biosystem, USA). qPCR was carried out in 16 µL total volumes containing 1X Eva Green mix, 0.5 pmol forward and reverse primer (Table 1) and 2 µL cDNA. Cycling conditions were as reported previously (21).

Table1. *Primers used in the gene expression analysis.

Gene	Sequence (5'-3')	Band size (bp)
16S rRNA-f **16S rRNA-r**	CCA GGA TTT GAT YMT GGC GAA GGA GGT GWT CCA DCC	532
marA-f ***marA-r***	TTA GGC CAA TAC ATC CGC AG AAG GTT CGG GTC AGA GTT TG	128
marR-f ***marR-r***	TGT AAA GGC TGG GTG GAA AG GTT AAT TCT TGG TGC AGG TCC	134
ompF-f ***ompF-r***	GGTGTTGGCGGTTCTATCAG TTCTTGCAGGTTGGTACGG	87
acrA-f ***acrA-r***	CAT TGG TAC AGA ACG GTC AGG GTT CTC TTG TTT CAG CGT GC	140
acrB-f ***acrB-r***	TTC CAT CTT CGC CAG TTC AG TCA TCG CAG AGT TTA ACG GC	113
tolC-f ***tolC-r***	CGG GAT TTC TGA CAC CTC TTA TAG ACC TGC GAG TTA ACC ATT CC	144

*Primer sequences were prepared for previous study (22).
(https://eu.idtdna.com/scitools/Applications/RealTimePCR/)

Statistical analysis

The influences of hormones on the growth of standard strains were detected by two-way ANOVA Bonferroni post-test. Additionally, gene expression levels (u/down regulations) were calculated using the one-way ANOVA unpaired t-test.

RESULTS

Alterations on Growth

The growth of *E. coli* SPC105 was shown to be affected in the presence of NE depending on hormone concentrations. The presence of low-level NE significantly decreased ($p<0.001$) the growth of the strain; however the growth was significantly increased ($p<0.001$) in the presence of both medium and high concentrations of NE after 4, 6 and 24 hours incubation (Figure 1).

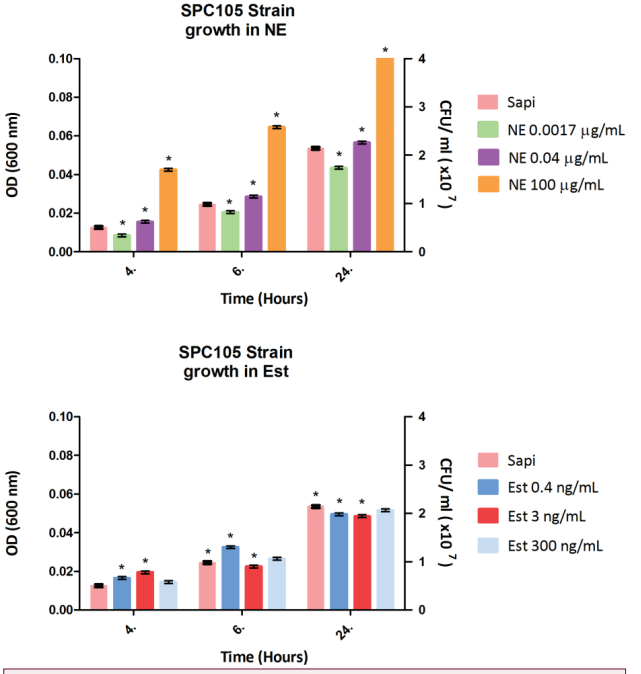

Figure 1. Effects of different concentrations of NE and Est on growth of *E. coli* SPC105. SAPI without any of hormones was used as control.
Growth alterations were performed using a two-way ANOVA Bonferroni post–test; *: $p<0.001$

Both low and medium concentrations of Est enhanced the growth of the strain significantly at the 4- and 6-hour periods ($p<0.001$). But in contrast, when the incubation time was extended, the growth was shown to be reduced by low and medium levels of Est at 24 hours incubation ($p<0.001$). A high level of Est was shown to have no influence on growth of *E. coli* SPC105 ($p>0.05$) (Figure 1).

Alterations on Gene Expressions

High quality total RNAs with $\Delta_{260/280}$ ~1.9-2.0 were obtained using a commercial total RNA isolation kit. Converted RNAs were subjected to qPCR and Rt-PCR assays. The melting scores and E values ranged between 90-100% and 1.8-2.1 respectively. These values showed that qPCRs were run efficiently. The minimum and maximum Cp values ranged between 20.84-27.80, 21.17-28.20, 20.27-27.17, 20.59-26.86, 20.74-27.48, 20.06-26.26 and 14.80-18.82 *marA*, *marR*, *acrA*, *acrB*, *tolC*, *ompF*

and 16S rRNA genes, respectively. $2^{-\Delta\Delta CT}$ values changed between 2.53±0.23-13.92±2.44, 2.96±0.16-15.48±1.3, 2.18±0.22-14.09±1.7, 1.85±0.004-12.44±0.74, 2.12±0.17-11.91±0.99 and 1.39±0.04-12.27±1.12 for *marA*, *marR*, *acrA*, *acrB*, *tolC* and *ompF* genes in different experimental conditions.

Fold changes in six target genes' expressions

Expressions of all six genes were found to have increased in the presence of both NE and Est in all concentrations. Significant differences were detected between the control and included hormones in SAPI for each gene ($p<0.05$ – $p<0.001$) (Figure 2).

DISCUSSION

Bacteria maintain a community behavior and adapt to their own habitats (host) via various chemical signaling pathways. These molecules which are known as quorum sensing molecules (QS) are essential for survival and optimizing of their

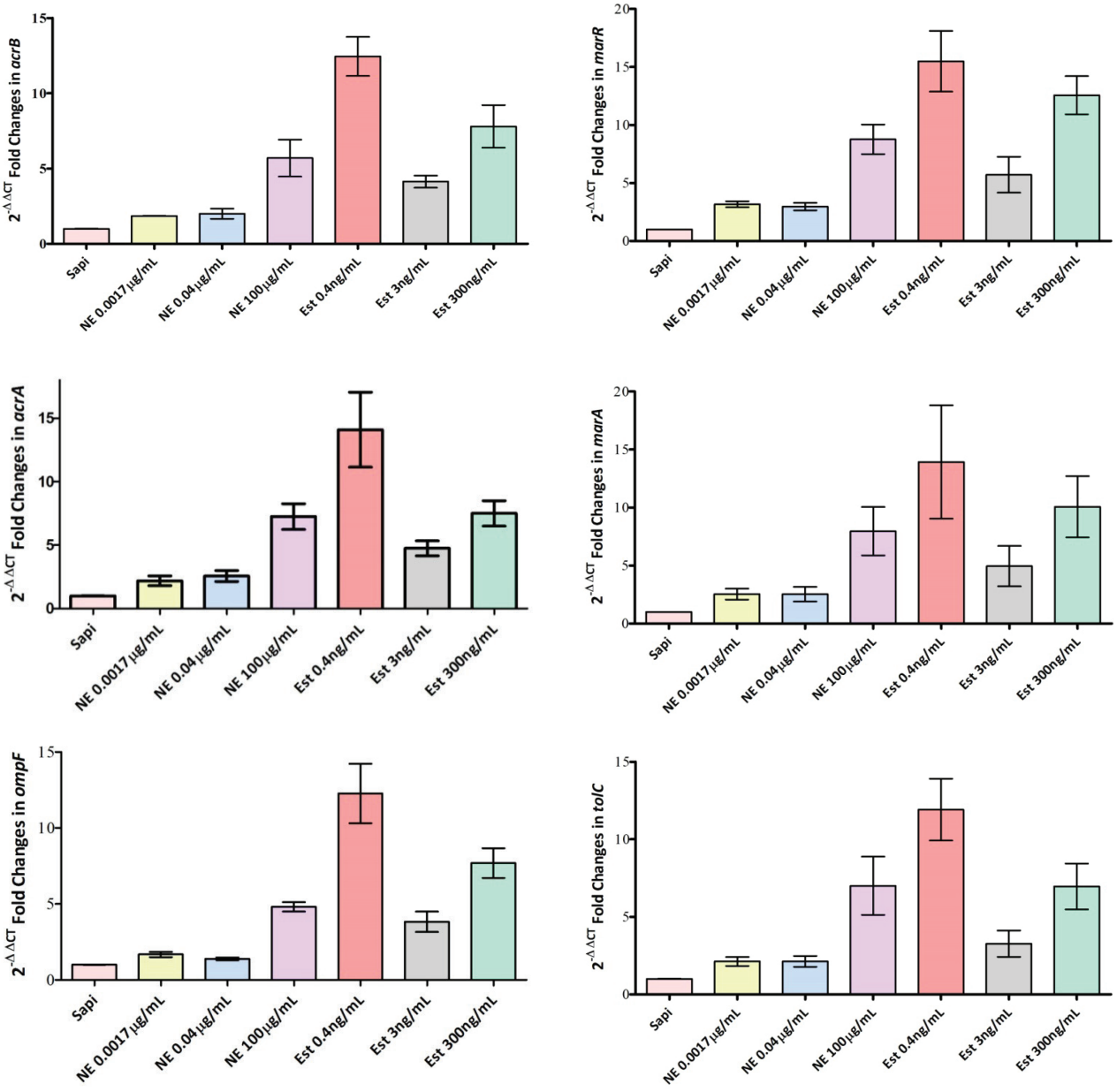

Figure 2. Fold-changes in *mar* expressions on mRNA transcriptional level.
Gene expression levels were determined using the one-way ANOVA unpaired t-test. To determine the effects of hormones, SAPI without NE/Est was used as a control. Alterations of all genes' expressions were found to be statistically significant ($p<0.05$ – $p<0.001$).

gene expressions, virulence mechanisms, and antibiotic resistance (23-25). It is well known that, not only bacteria-bacteria communication but also bacteria-host communication is very essential, this is known as inter kingdom signaling. According to previous studies, it is clear that to maintain this cell-to-cell communication, bacteria may use certain host factors such as hormones. Stress for instance, is very important to generate an unfavorable condition for the host which leads to them becoming more susceptible to infections and even the immune functions are induced. Therefore, an emergent research area, "Microbial Endocrinology", has gained importance to investigate how these hormones could affect the biological properties of bacteria (1,18). In our study, the possible effects of NE (a stress hormone) and Est (a gender hormone) on growth and expressions of certain genes associated with mar regulon of *E. coli* SPC105 were investigated. These were chosen as examples of human hormones with different effects and they also carry phenolic groups in their structure.

Previous investigations have also shown that the enteric nervous system in mammalian hosts is the primary site for NE to reproduce and to be utilized. The gut is the main habitat for NE; that's why most of the studies focusing on host-microbe communication target *E. coli* strains (1,26). Catecholamines such as NE, regulate the biological properties of microorganisms by the iron accessing mechanism (1). It is very clear that sex hormones such as Est and progesterone also influence the growth and gene regulation mechanisms of bacteria because they replace vitamin K (1,5,27). Also, they have proven to have protective activity against infections via induction of bactericidal activities of proinflammatory cells and innate and adaptive immune responses of the host (27-29). Previous investigations have shown that these host derived hormones could also be metabolized by fecal bacteria to use them as carbon and energy sources (1,27,29). Numerous studies have reported that host hormones have certain effects on the various gene expressions of bacteria related with enzyme metabolism, virulence, iron uptake systems, stress responses and antibiotic resistance as mentioned above (21,30-33).

The influence of hormones on the growth of various bacteria has been shown in many studies (1,4,5,18,21,32-35). Gümüş et al. investigated the SPC105 strain to show the possible effects of human insulin and glucose on growth and expression levels of mar operon (22). They have shown that in a rich culture medium (TSB), the growth of SPC105 was shown to have decreased in the presence of 200µU insulin+0.1% glucose and 0.1% glucose; however, 20 µU insulin and 200 µU insulin did not affect the growth of bacterium. Therefore, it may be concluded that, in standard, rich medium the effects of hormones cannot be detected effectively which led us to select serum-SAPI in this study.

In the present study we detected alterations of growth depending on hormone types and/or concentrations. NE for instance, significantly enhanced the growth of the SPC105 *E. coli* strain with medium and high-level concentrations tested at the four, six and 24 hours periods. According to previous studies, this effect may be related to iron acquisition from the transferrin (serum iron-binding protein) and transferring to the bacteria (1,26,33,36). Although Kornman and Loeshe reported that Est could inhibit the growth of Bacteroides species (5), our results showed enhancement of growth in the presence of low and medium Est concentrations at 4 and 6 hours; however, when the incubation time was increased to 24 hours, the growth was found to be reduced. We found no induction on the growth of bacteria in the presence of high-level Est. Therefore, we suggest that growth alterations could be associated with exposure time, hormone concentration, and the strain tested.

Previous studies have shown that host hormones could affect the expression levels of antimicrobial resistance genes in *Candida* species, *Staphylococcus aureus, E. coli, Edwardsiella* (37-41). Plotkin and Konakieva suggested that microorganisms sense different environmental signals and develop various genetic modifications such as chromosomal mutations or up regulation of efflux pumps to defend themselves (37). Another previous study focusing on expression levels of *mar* operon located in SPC105 strain which was examined in the present study showed a significant down regulation in the expression levels of *marA* and *marR* in the presence of 200 µU/mL insulin, 0.1% glucose and 200 µU/ mL insulin + 0.1% glucose. The expression of *acrA* was also decreased in the presence of 200 µU/mL insulin (22) which may be due to a reduction in growth of bacterium as mentioned above. Cohen et al. showed the significant six fold increase in β-galactosidase activity in the *E. coli* SPC105 strain in the presence of salicylate and because it carries mar promoter-lacZ fusion on the chromosome, this increase also denotes the induction of *mar* operon (42). The expression level of mar operon and growth alterations were also investigated in a study by Maira-Litrán et al. by comparing two different culture media (LB and CDM; nutrient rich and poor, respectively) in planktonic and biofilm conditions. They showed that, β-galactosidase levels were higher in CDM rather than LB. When the E. coli SPC105 was grown in CDM medium, via biofilm conditions, the expression of mar was not induced (43). In the present study, gene expression levels of six target genes (*marA*, *marR*, *ompF*, *acrA*, *acrB* and *tolC*) associated with chromosomal multiple antibiotic resistance were found to be increased in the presence of both NE and Est with all three concentrations. It is known that MarR suppresses *marRAB* operon, but *marRAB* transcription occurs if MarR suppression is removed which may be due to mutations or presence of phenolic compounds, oxidative stress inducers and antibiotics (11,16). In our study we assumed that these hormones enhanced the expression of *marR*, but in the meantime MarR may bind to NE and Est which can lead to derepression of MarR.

Multidrug resistance is associated with *marRAB* operon due to the activation of MarA, which yields both up regulations of the AcrAB-TolC efflux pump and down regulation of the outer membrane porin OmpF (10,42,44). In the present study, the up regulations of *acrA*, *acrB*, and *tolC* genes were shown to be con-

sistent with previous studies; but on the other hand our results have also suggested that, hormones affected the expression levels of *ompF* independently from *marA*.

In conclusion, our results constitute new data on the possible effects of these two mammalian hormones (NE and Est) on the growth and on mar operon's expressions on the transcriptional level in the *E. coli* SPC105 strain. It may be concluded that these effects depended on hormone concentrations, exposure time, medium used and strain tested. Also, during exposure to certain environmental factors, genes associated with *mar* operon could be affected independently from each other (*marR-marA*) and from the mechanism identified in previous studies (12,13). As a future perspective, it seems that we are going to look for an answer as to whether, during an infection, hormones as one of the environmental factors in a human host could lead to multi drug resistance in different type of bacteria.

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REFERENCES

1. Lyte M, Freestone PP. Microbial Endocrinology: Inter kingdom signaling in Infectious Disease and Health. New York: Springer; 2010. [CrossRef]
2. LeRoith D, Shiloach J, Roth J, Lesniak MA. Insulin or a closely related molecule is native to Escherichia coli. Journal of Biological Chemistry 1981; 256(13): 6533-6. [CrossRef]
3. Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol 2001; 55: 165-99. [CrossRef]
4. Plotkin BJ, Viselli SM. Effect of insulin on microbial growth. Curr Microbiol 2000; 41: 60-4. [CrossRef]
5. Kornman KS, Loesche WJ. Effects of estradiol and progesterone on Bacteroides melaninogenicus and Bacteroides gingivalis. Infect and Immun 1982; 35(1): 256-63. [CrossRef]
6. Delcenserie V, LaPointe G, Charaslertrangsi T, Rabalski A, Griffiths MW. Glucose decreases virulence gene expression of Escherichia coli O157: H7. J Food Protect 2012; 75(4): 748-52. [CrossRef]
7. Ruiz C, Levy SB. Many chromosomal genes modulate MarA-mediated multidrug resistance in Escherichia coli. Antimicrobiol Agents Chemother 2010; 54(5): 2125-34. [CrossRef]
8. Randall LP, Woodward MJ. The multiple antibiotic resistance (mar) locus and its significance. Res Vet Sci 2002; 72: 87-93. [CrossRef]
9. Kunonga NI, Sobieski RJ, Crupper SS. Prevalence of the multiple antibiotic resistance operon (marRAB) in the genus Salmonella. FEMS Microbiol Lett 2000; 187(2): 155-60. [CrossRef]
10. Vinué L, McMurry LM, Levy SB. The 216-bp marB gene of the marRAB operon in Escherichia coli encodes a periplasmic protein which reduces the transcription rate of marA. FEMS Microbiol Lett 2013; 345(1): 49-55. [CrossRef]
11. Weston N, Sharma P, Ricci V, Piddock LJ. Regulation of the AcrAB-TolC efflux pump in Enterobacteriaceae. Res Microbiol 2018; 169(7-8): 425-31. [CrossRef]
12. Alekshun MN. Levy SB. The mar regulon: multiple resistances to antibiotics and other toxic chemicals. Trends Microbiol 1999; 7(10): 410-13. [CrossRef]
13. Alekshun MN, Levy SB. The Escherichia coli mar locus-Antibiotic resistance and more. ASM News 2004; 70(10): 451-56.
14. Lankester A, Ahmed S, Lamberte LE, Kettles RA, Grainger DC. The Escherichia coli multiple antibiotic resistance activator protein represses transcription of the lac operon. Biochem Society Transact 2019; 47(2): 671-77. [CrossRef]
15. Zgurskaya HI, Krishnamoorthy G, Ntreh A, Lu S. Mechanism and function of the outer membrane channel TolC in multidrug resistance and physiology of enterobacteria. Frontiers Microbiol 2011; 2: 189. [CrossRef]
16. Misra R, Bavro VN. Assembly and transport mechanism of tripartite drug efflux systems. Biochimica et Biophysica Acta (BBA) Proteins Proteomics 2009; 1794(5): 817-25. [CrossRef]
17. Rickard AH, Lindsay S, Lockwood GB, Gilbert P. Induction of the mar operon by miscellaneous groceries. J Appl Microbiol 2004; 97(5): 1063-68. [CrossRef]
18. Lyte M, Ernst S. Catecholamine induced growth of gram negative bacteria. Life Sci 1992; 50(3): 203-12. [CrossRef]
19. Zhang X, Essmann M, Burt ET, Larsen B. Estrogen effects on Candida albicans: a potential virulence-regulating mechanism. J Infect Dis 2000; 181(4): 1441-46. [CrossRef]
20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 2001; 25: 402-08. [CrossRef]
21. Gumus D, Yoruk E, Kalayci-Yuksek F, Uz G, Topal-Sarikaya A, Ang-Kucuker M. The effects of insulin and glucose on different characteristics of a UPEC: alterations in growth rate and expression levels of some virulence genes. Clin Lab 2017; 63(10): 1589-97. [CrossRef]
22. Gümüş D, Yüksek FK, Uz G, Bayraktar A, Köseoğlu P, Ofluoğlu Y, et al. The effects of insulin and glucose on growth and expression of mar regulon in E. coli. Turkish J Clin Lab 2019; 10(2): 209-16.
23. Williams P. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiol 2007; 153(12): 3923-38. [CrossRef]
24. Sperandio V. Torres AG, Jarvis B, Nataro JP, and Kaper JB. Bacteria-host communication: the language of hormones. Proc Natl Acad Sci USA 2003; 100: 8951-6. [CrossRef]

25. Hughes DT, Sperandio V. Inter-kingdom signalling: communication between bacteria and their hosts. Nature Reviews Microbiology 2008; 6(2): 111-20. [CrossRef]
26. Lyte M, Freestone PP, Neal CP, Olson BA, Haigh RD, Bayston R, et al. Stimulation of Staphylococcus epidermidis growth and biofilm formation by catecholamine inotropes. Lancet 2003; 361 (9352): 130-5. [CrossRef]
27. García-Gómez E, González-Pedrajo B, Camacho-Arroyo I. Role of sex steroid hormones in bacterial-host interactions. BioMed Res International 2013; 2013: 928290. [CrossRef]
28. Saia RS, Garcia FM, Cárnio EC. Estradiol protects female rats against sepsis induced by Enterococcus faecalis improving leukocyte bactericidal activity. Steroids 2015; 102: 17-26. [CrossRef]
29. Patt MW, Conte L, Blaha M, Plotkin BJ. Steroid hormones as interkingdom signaling molecules: Innate immune function and microbial colonization modulation. AIMS Molecul Sci 2018; 5(1): 117-30. [CrossRef]
30. Plotkin BJ, Roose RJ, Erikson Q, Viselli SM. Effect of androgens and glucocorticoids on microbial growth and antimicrobial susceptibility. Curr Microbiol 2003; 47(6): 514-20. [CrossRef]
31. Jerse AE, Wu H, Packiam M, Vonck RA, Begum A, Garvin LE. Estradiol-treated female mice as surrogate hosts for Neisseria gonorrhoeae genital tract infections. Frontiers Microbiol 2011; 2: 107. [CrossRef]
32. Intarak N, Muangsombut V, Vattanaviboon P, Stevens MP, Korbsrisate S. Growth, motility and resistance to oxidative stress of the melioidosis pathogen Burkholderia pseudomallei are enhanced by epinephrine. Pathogens Dis 2014; 72(1): 24-31. [CrossRef]
33. Li W, Lyte M, Freestone PP, Ajmal A, Colmer-Hamood JA, Hamood AN. Norepinephrine represses the expression of toxA and the siderophore genes in Pseudomonas aeruginosa. FEMS Microbiol Lett 2009; 299(1): 100-09. [CrossRef]
34. Yokoyama M, Hinode D, Masuda K, Yoshioka M, Grenier D. Effect of female sex hormones on Campylobacter rectus and human gingival fibroblasts. Oral Microbiol Immunol 2005; 20(4): 239-43. [CrossRef]
35. Gonullu N, Kucukbasmaci O, Buyukbaba-Boral O, Ang-Kucuker M. The influence of glucose added urine on the in vitro antimicrobial activity of various antibiotics. Indian J Med Res 2008; 128(5): 663-5.
36. Roberts A, Matthews JB, Socransky SS, Freestone PPE, Williams PH, Chapple ILC. Stress and the periodontal diseases: effects of catecholamines on the growth of periodontal bacteria in vitro. Oral Microbiol Immunol 2002; 17(5): 296-303. [CrossRef]
37. Plotkin BJ, Konakieva MI. Attenuation of antimicrobial activity by the human steroid hormones. Steroids 2017; 128: 120-7. [CrossRef]
38. Micheli MD, Bille J, Schueller C, Sanglard D. A common drug-responsive element mediates the upregulation of the Candida albicans ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Molecular Microbiol 2002; 43(5): 1197-214. [CrossRef]
39. Banerjee D, Martin N, Nandi S, Shukla S, Dominguez A, Mukhopadhyay G, Prasad R. A genome-wide steroid response study of the major human fungal pathogen Candida albicans. Mycopathologia 2007; 164(1): 1-17. [CrossRef]
40. Peng B, Ma YM, Zhang JY, Li H. Metabolome strategy against Edwardsiella tarda infection through glucose-enhanced metabolic modulation in tilapias. Fish & Shellfish Immunol 2015; 45(2): 869-76. [CrossRef]
41. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 2011; 473(7346): 216-20. [CrossRef]
42. Cohen SP, Levy SB, Foulds J, Rosner JL. Salicylate induction of antibiotic resistance in Escherichia coli: activation of the mar operon and a mar-independent pathway. J Bacteriol 1993; 175(24): 7856-62. [CrossRef]
43. Maira-Litrán T, Allison DG, Gilbert P. Expression of the multiple antibiotic resistance operon (mar) during growth of Escherichia coli as a biofilm. J Appl Microbiol 2000; 88(2): 243-7. [CrossRef]
44. Chubiz LM, Rao CV. Role of the mar-sox-rob regulon in regulating outer membrane porin expression. J Bacteriol 2011; 193(9): 2252-60. [CrossRef]

EXPERIMED

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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 languages of the journal are Turkish and English.

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

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

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

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Processing and publication are free of charge with the journal. No fees are requested from the authors at any point throughout the evaluation and publication process. All manuscripts must be submitted via the online submission system, which is available at http://experimed.istanbul.edu.tr/en/_.The journal guidelines, technical information, and the required forms are available on the journal's web page.

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AMAÇ VE KAPSAM

Experimed; İstanbul Üniversitesi Aziz Sancar Deneysel Araştırma Enstitüsü'nün çift-kör hakemli, elektronik, uluslararası, açık erişimli bilimsel yayın organıdır. Dergi Nisan, Ağustos ve Aralık aylarında olmak üzere, yılda 3 sayı olarak yayınlanır. Derginin yayın dili Türkçe ve İngilizce'dir.

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Abstract: A Turkish and an English abstract should be submitted with all submissions except for Letters to the Editor. Submitting a Turkish abstract is not compulsory for international authors. The abstract of Original Articles should be structured with subheadings (Objective, Material and Method, Results, and Conclusion). Please check Table 1 below for word count specifications.

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

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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

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Özgünlük, yüksek bilimsel kalite ve atıf potansiyeli bir makalenin yayına kabulü için en önemli kriterlerdir. Gönderilen yazıların daha önce başka bir elektronik ya da basılı dergide, kitapta veya farklı bir mecrada sunulmamış ya da yayınlanmamış olması gerekir. Daha önce başka bir dergiye gönderilen ancak yayına kabul edilmeyen yazılar hakkında dergi önceden bilgilendirilmelidir. Bu yazıların eski hakem raporlarının Yayın Kuruluna gönderilmesi değerlendirme süresinin hızlanmasını sağlayacaktır. Toplantılarda sunulan çalışmalar için, sunum yapılan organizasyonun tam adı, tarihi, şehri ve ülkesi belirtilmelidir.

Değerlendirme Süreci
Experimed'e gönderilen tüm makaleler çift-kör hakem değerlendirme sürecinden geçmektedir. Tarafsız değerlendirme sürecini sağlamak için her makale alanlarında uzman en az iki dış-bağımsız hakem tarafından değerlendirilir. Dergi Yayın Kurulu üyeleri tarafından gönderilecek makalelerin değerlendirme süreçleri, davet edilecek dış bağımsız editörler tarafından yönetilecektir. Bütün makalelerin karar verme süreçlerinde nihai karar yetkisi Baş Editör'dedir.

Etik İlkeler
Klinik ve deneysel çalışmalar, ilaç araştırmaları ve bazı olgu sunumları için World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013, www.wma.net) çerçevesinde hazırlanmış Etik Komisyon raporu gerekmektedir. Gerekli görülmesi halinde Etik Komisyon raporu veya eşdeğeri olan resmi bir yazı yazarlardan talep edilebilir. İnsanlar üzerinde yapılmış deneysel çalışmaların sonuçlarını bildiren yazılarda, çalışmanın yapıldığı kişilere uygulanan prosedürlerin niteliği tümüyle açıklandıktan sonra, onaylarının alındığına ilişkin bir açıklamaya metin içinde yer verilmelidir. Hayvanlar üzerinde yapılan çalışmalarda ise ağrı, acı ve rahatsızlık verilmemesi için yapılmış olanlar açık olarak makalede belirtilmelidir. Hasta onamları, Etik Kurul raporun alındığı kurumun adı, onay belgesinin numarası ve tarihi ana metin dosyasında yer alan Yöntemler başlığı altında yazılmalıdır. Hastaların kimliklerinin gizliliğini korumak yazarların sorumluluğundadır. Hastaların kimliğini açığa çıkarabilecek fotoğraflar için hastadan ya da yasal temsilcilerinden alınan imzalı izinlerin de gönderilmesi gereklidir.

Dergiye gönderilen makaleler, hakem değerlendirme sürecinde ya da yayına hazırlık aşamasında herhangi bir noktada bir benzerlik tespit yazılımı (CrossCheck, iThenticate) tarafından taranmaktadır. Cümleler ve ifadeler yazar olarak size ait olsa dahi, metnin daha önce yayınlanan verilerle kabul edilemez bir benzerliği olmalıdır.

Başkalarının önceki çalışmalarını (veya kendi çalışmalarınızı) tartışırken, lütfen materyali her durumda doğru bir şekilde alıntıladığınızdan emin olunuz.

Yayın Kurulu, dergimize gönderilen çalışmalar hakkındaki intihal, atıf manipülasyonu ve veri sahteciliği iddia ve şüpheleri karşısında COPE kurallarına uygun olarak hareket edecektir.

Yazarlık
Yazar olarak listelenen herkesin ICMJE (www.icmje.org) tarafından önerilen yazarlık kriterlerini karşılaması gerekmektedir. ICMJE, yazarların aşağıdaki 4 kriteri karşılamasını önermektedir:

1. Çalışmanın konseptine/tasarımına; ya da çalışma için verilerin toplanmasına, analiz edilmesine ve yorumlanmasına önemli katkı sağlamış olmak; VE
2. Yazı taslağını hazırlamış ya da önemli fikirsel içeriğin eleştirel incelemelerini yapmış olmak; VE
3. Yazının yayından önceki son halini gözden geçirmiş ve onaylamış olmak; VE
4. Çalışmanın herhangi bir bölümünün geçerliliği ve doğruluğuna ilişkin soruların uygun şekilde soruşturulduğunun ve çözümlendiğinin garantisini vermek amacıyla çalışmanın her yönünden sorumlu olmayı kabul etmek.

Bir yazar, çalışmada katkı sağladığı kısımların sorumluluğunu almasına ek olarak, diğer yazarların çalışmanın hangi kısımlarından sorumlu olduğunu da teşhis edebilmelidir. Ayrıca, yazarlar birbirlerinin katkılarının bütünlüğüne güven duymalılardır.

Yazar olarak belirtilen her kişi yazarlığın dört kriterini karşılamalıdır ve bu dört kriteri karşılayan her kişi yazar olarak tanımlanmalıdır. Dört kriterin hepsini karşılamayan kişilere makalenin başlık sayfasında teşekkür edilmelidir.

Yazarlık haklarına uygun hareket etmek ve hayalet ya da lütuf yazarlığın önlenmesini sağlamak amacıyla sorumlu yazarlar makale yükleme sürecinde http://experimed.istanbul.edu.tr/tr/_ adresinden erişebilinen Yazar Katkı Formu'nu imzalamalı ve taranmış versiyonunu yazıyla birlikte göndermelidir. Yayın Kurulu'nun gönderilen bir makalede "lütuf yazarlık" olduğundan şüphelenmesi durumunda söz konusu makale değerlendirme yapılmaksızın reddedilecektir. Makale gönderimi kapsamında; sorumlu yazar makale gönderim ve

değerlendirme süreçleri boyunca yazarlık ile ilgili tüm sorumluluğu kabul ettiğini bildiren kısa bir ön yazı göndermelidir.

Çıkar Çatışması
Experimed; gönderilen makalelerin değerlendirme sürecine dahil olan yazarların ve bireylerin, potansiyel çıkar çatışmasına ya da önyargıya yol açabilecek finansal, kurumsal ve diğer ilişkiler dahil mevcut ya da potansiyel çıkar çatışmalarını beyan etmelerini talep ve teşvik eder.

Bir çalışma için bir birey ya da kurumdan alınan her türlü finansal destek ya da diğer destekler Yayın Kurulu'na beyan edilmeli ve potansiyel çıkar çatışmalarını beyan etmek amacıyla ICMJE Potansiyel Çıkar Çatışmaları Formu katkı sağlayan tüm yazarlar tarafından ayrı ayrı doldurulmalıdır. Editörler, yazarlar ve hakemler ile ilgili potansiyel çıkar çatışması vakaları derginin Yayın Kurulu tarafından COPE ve ICMJE rehberleri kapsamında çözülmektedir.

Derginin Yayın Kurulu, itiraz ve şikayet vakalarını, COPE rehberleri kapsamında işleme almaktadır. Yazarlar, itiraz ve şikayetleri için doğrudan Editöryel Ofis ile temasa geçebilirler. İhtiyaç duyulduğunda Yayın Kurulu'nun kendi içinde çözemediği konular için tarafsız bir temsilci atanmaktadır. İtiraz ve şikayetler için karar verme süreçlerinde nihai kararı Baş Editör verecektir.

Telif ve Lisans
Yazarlar dergide yayınlanan çalışmalarının telif hakkına sahiptirler ve çalışmaları Creative Commons Atıf-GayrıTicari 4.0 Uluslararası (https://creativecommons.org/licenses/by-nc/4.0/deed.tr" CC BY-NC 4.0) https://creativecommons.org/licenses/by-nc/4.0/deed.tr olarak lisanslıdır. CC BY-NC 4.0 lisansı, eserin ticari kullanım dışında her boyut ve formatta paylaşılmasına, kopyalanmasına, çoğaltılmasına ve orijinal esere uygun şekilde atıfta bulunmak kaydıyla yeniden düzenleme, dönüştürme ve eserin üzerine inşa etme dâhil adapte edilmesine izin verir.

Açık Erişim İlkesi
Dergi açık erişimlidir ve derginin tüm içeriği okura ya da okurun dahil olduğu kuruma ücretsiz olarak sunulur. Okurlar, ticari amaç haricinde, yayıncı ya da yazardan izin almadan dergi makalelerinin tam metnini okuyabilir, indirebilir, kopyalayabilir, arayabilir ve link sağlayabilir. Bu HYPERLINK "https://www.budapestopenaccessinitiative.org/translations/turkish-translation" BOAI açık erişim tanımıyla uyumludur.

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Sorumluluk Reddi
Dergide yayınlanan makalelerde ifade edilen görüşler ve fikirler Experimed, Baş Editör, Editörler, Yayın Kurulu ve Yayıncı'nın değil, yazar(lar)ın bakış açılarını yansıtır. Baş Editör, Editörler, Yayın Kurulu ve Yayıncı bu gibi durumlar için hiçbir sorumluluk ya da yükümlülük kabul etmemektedir. Yayınlanan içerik ile ilgili tüm sorumluluk yazarlara aittir.

MAKALE HAZIRLAMA

Makaleler, ICMJE-Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals (updated in December 2015 - http://www.icmje. org/icmje-recommendations.pdf) ile uyumlu olarak hazırlanmalıdır. Randomize çalışmalar CONSORT, gözlemsel çalışmalar STROBE, tanısal değerli çalışmalar STARD, sistematik derleme ve meta-analizler PRISMA, hayvan deneyli çalışmalar ARRIVE ve randomize olmayan davranış ve halk sağlığıyla ilgili çalışmalar TREND kılavuzlarına uyumlu olmalıdır.

Makaleler sadece http://experimed.istanbul.edu.tr/tr/_ adresinde yer alan derginin online makale yükleme ve değerlendirme sistemi üzerinden gönderilebilir. Diğer mecralardan gönderilen makaleler değerlendirilmeye alınmayacaktır.

Gönderilen makalelerin dergi yazım kurallarına uygunluğu ilk olarak Editöryel Ofis tarafından kontrol edilecek, dergi yazım kurallarına uygun hazırlanmamış makaleler teknik düzeltme talepleri ile birlikte yazarlarına geri gönderilecektir.

Yazarların; Telif Hakkı Anlaşması Formu, Yazar Katkı Formu ve ICMJE Potansiyel Çıkar Çatışmaları Formu'nu (bu form, tüm yazarlar tarafından doldurulmalıdır) ilk gönderim sırasında online makale sistemine yüklemeleri gerekmektedir. Bu formlara http://experimed.istanbul.edu.tr/tr/_ adresinden erişilebilmektedir.

Başlık sayfası: Gönderilen tüm makalelerle birlikte ayrı bir başlık sayfası da gönderilmelidir. Bu sayfa;

- Makalenin başlığını ve 50 karakteri geçmeyen kısa başlığını,
- Yazarların isimlerini, kurumlarını, ORCID numaralarını ve eğitim derecelerini,
- Finansal destek bilgisi ve diğer destek kaynakları hakkında detaylı bilgiyi,
- Sorumlu yazarın ismi, adresi, telefonu (cep telefonu dahil ve e-posta adresini,
- Makale hazırlama sürecine katkıda bulunan ama yazarlık kriterlerini karşılamayan bireylerle ilgili bilgileri içermelidir.

Özet: Editöre Mektup türündeki yazılar dışında kalan tüm makalelerin Türkçe ve İngilizce özetleri olmalıdır. rijinal Araştırma makalelerinin özetleri "Amaç", "Gereç ve Yöntem", "Bulgular" ve "Sonuç" alt başlıklarını içerecek biçimde hazırlanmalıdır.

Anahtar Sözcükler: Tüm makaleler en az 3 en fazla 6 anahtar kelimeyle birlikte gönderilmeli, anahtar sözcükler özetin hemen altına yazılmalıdır. Kısaltmalar anahtar sözcük olarak kullanılmamalıdır. Anahtar sözcükler National Library of Medicine (NLM) tarafından hazırlanan Medical Subject Headings (MeSH) veritabanından seçilmelidir.

Makale Türleri
Orijinal Araştırma: Ana metin "Giriş", "Gereç ve Yöntem", "Bulgular" ve "Tartışma" alt başlıklarını içermelidir. Özgün Araştırmalarla ilgili kısıtlamalar için lütfen Tablo 1'i inceleyiniz.

Sonucu desteklemek için istatiksel analiz genellikle gereklidir. İstatistiksel analiz, tıbbi dergilerdeki istatistik verilerini bildirme kurallarına göre yapılmalıdır (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). İstatiksel analiz ile ilgili bilgi, Yöntemler bölümü içinde ayrı bir alt başlık olarak yazılmalı ve kullanılan yazılım kesinlikle tanımlanmalıdır.

Birimler, uluslararası birim sistemi olan International System of Units (SI)'a uygun olarak hazırlanmadır.

Editöryel Yorum: Dergide yayınlanan bir araştırmanın, o konunun uzmanı olan veya üst düzeyde değerlendirme yapan bir hakemi tarafından kısaca yorumlanması amacını taşımaktadır. Yazarları, dergi tarafından seçilip davet edilir. Özet, anahtar sözcük, tablo, şekil, resim ve diğer görseller kullanılmaz.

Derleme: Yazının konusunda birikimi olan ve bu birikimleri uluslararası literatüre yayın ve atıf sayısı olarak yansımış uzmanlar tarafından hazırlanmış yazılar değerlendirmeye alınır. Yazarları dergi tarafından da davet edilebilir. Bir bilgi ya da konunun klinikte kullanılması için vardığı son düzeyi anlatan, tartışan, değerlendiren ve gelecekte yapılacak olan çalışmalara yön veren bir formatta hazırlanmalıdır. Ana metin "Giriş", "Klinik ve Araştırma Etkileri" ve "Sonuç" bölümlerini içermelidir. Derleme türündeki yazılarla ilgili kısıtlamalar için lütfen Tablo 1'i inceleyiniz.

Olgu Sunumu: Olgu sunumları için sınırlı sayıda yer ayrılmakta ve sadece ender görülen, tanı ve tedavisi güç olan hastalıklarla ilgili, yeni bir yöntem öneren, kitaplarda yer verilmeyen bilgileri yansıtan, ilgi çekici ve öğretici özelliği olan olgular yayına kabul edilmektedir. Ana metin; "Giriş", "Olgu Sunumu", "Tartışma" ve "Sonuç" alt başlıklarını içermelidir. Olgu Sunumlarıyla ilgili kısıtlamalar için lütfen Tablo 1'i inceleyiniz.

Editöre Mektup: Dergide daha önce yayınlanan bir yazının önemini, gözden kaçan bir ayrıntısını ya da eksik kısımlarını tartışabilir. Ayrıca derginin kapsamına giren alanlarda okurların ilgisini çekebilecek konular ve özellikle eğitici olgular hakkında da Editöre Mektup formatında yazılar yayınlanabilir. Okuyucular da yayınlanan yazılar hakkında yorum içeren Editöre Mektup formatında yazılarını sunabilirler. Özet, anahtar sözcük, tablo, şekil, resim ve diğer görseller kullanılmaz. Ana metin alt başlıksız olmalıdır. Hakkında mektup yazılan yayına ait cilt, yıl, sayı, sayfa numaraları, yazı başlığı ve yazarların adları açık bir şekilde belirtilmeli, kaynak listesinde yazılmalı ve metin içinde atıfta bulunulmalıdır.

Tablolar
Tablolar ana dosyaya eklenmeli, kaynak listesi sonrasında sunulmalı, ana metin içerisindeki geçiş sıralarına uygun olarak numaralandırılmadır. Tabloların üzerinde tanımlayıcı bir başlık yer almalı ve tablo içerisinde geçen kısaltmaların açılımları tablo altına tanımlanmalıdır. Tablolar Microsoft Office Word dosyası içinde "Tablo Ekle" komutu kullanılarak hazırlanmalı ve kolay okunabilir şekilde düzenlenmelidir. Tablolarda sunulan veriler ana metinde sunulan verilerin tekrarı olmamalı; ana metindeki verileri destekleyici nitelikte olmalılardır.

Resim ve Resim Altyazıları
Resimler, grafikler ve fotoğraflar (TIFF ya da JPEG formatında) ayrı dosyalar halinde sisteme yüklenmelidir. Görseller bir Word dosyası dokümanı ya da ana doküman içerisinde sunulmamalıdır. Alt birimlere ayrılan görseller olduğunda, alt birimler tek bir görsel içerisinde verilmemelidir. Her bir alt birim sisteme ayrı bir dosya olarak yüklenmelidir. Resimler alt birimleri belli etme amacıyla etiketlenmemelidir (a, b, c vb.). Resimlerde altyazıları desteklemek için kalın ve ince oklar, ok başları, yıldızlar, asteriksler ve benzer işaretler kullanılabilir. Makalenin geri kalanında olduğu gibi resimler de kör olmalıdır. Bu sebeple, resimlerde yer alan kişi ve kurum bilgileri de körleştirilmelidir. Görsellerin minimum çözünürlüğü 300DPI olmalıdır. Değerlendirme sürecindeki aksaklıkları önlemek için gönderilen bütün görsellerin çözünürlüğü net ve boyutu büyük (minimum boyutlar 100x100 mm) olmalıdır. Resim altyazıları ana metnin sonunda yer almalıdır.

Makale içerisinde geçen tüm kısaltmalar, ana metin ve özette ayrı ayrı olmak üzere ilk kez kullanıldıkları yerde tanımlanarak kısaltma tanımın ardından parantez içerisinde verilmelidir.

Ana metin içerisinde cihaz, yazılım, ilaç vb. ürünlerden bahsedildiğinde ürünün ismi, üreticisi, üretildiği şehir ve ülke bilgisini içeren ürün bilgisi parantez içinde verilmelidir; "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)".

Tüm kaynaklar, tablolar ve resimlere ana metin içinde uygun olan yerlerde sırayla numara verilerek atıf yapılmalıdır.

Özgün araştırmaların kısıtlamaları, engelleri ve yetersizliklerinden Sonuç paragrafı öncesi "Tartışma" bölümünde bahsedilmelidir.

Kaynaklar
Atıf yapılırken en son ve en güncel yayınlar tercih edilmelidir. Kaynakların doğruluğundan yazarlar sorumludur. Kaynaklar Vancouver referans stiline uygun olarak hazırlanmalıdır. Atıf yapılan erken çevrimiçi makalelerin DOI numaraları mutlaka sağlanmalıdır. Dergi

Tablo 1. Makale türleri için kısıtlamalar

Makale türü	Sözcük limiti	Özet sözcük limiti	Kaynak limiti	Tablo limiti	Resim limiti
Özgün Araştırma	3500	200 (Alt başlıklı)	30	6	7 ya da toplamda 15 resim
Derleme	5000	200	50	6	10 ya da toplamda 20 resim
Olgu Sunumu	1000	200	15	Tablo yok	10 ya da toplamda 20 resim
Editöre Mektup	500	Uygulanamaz	5	Tablo yok	Resim yok

EXPERIMED

isimleri Index Medicus/Medline/PubMed'de yer alan dergi kısaltmaları ile uyumlu olarak kısaltılmalıdır. Altı ya da daha az yazar olduğunda tüm yazar isimleri listelenmelidir. Eğer 7 ya da daha fazla yazar varsa ilk 6 yazar yazıldıktan sonra "et al" konulmalıdır. Ana metinde kaynaklara atıf yapılırken parantez içinde Arabik numaralar kullanılmalıdır. Farklı yayın türleri için kaynak stilleri aşağıdaki örneklerde sunulmuştur:

Dergi makalesi: Blasco V, Colavolpe JC, Antonini F, Zieleskiewicz L, Nafati C, Albanèse J, et al. Long-term outcome in kidney recipients from donors treated with hydroxyethylstarch 130/0.4 and hydroxyethylstarch 200/0.6. Br J Anaesth 2015; 115: 797-8.

Kitap bölümü: Sherry S. Detection of thrombi. In: Strauss HE, Pitt B, James AE, editors. Cardiovascular Medicine. St Louis: Mosby; 1974.p.273-85.

Tek yazarlı kitap: Cohn PF. Silent myocardial ischemia and infarction. 3rd ed. New York: Marcel Dekker; 1993.

Yazar olarak editör(ler): Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

Toplantıda sunulan yazı: 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.p.1561-5.

Bilimsel veya teknik rapor: Smith P. Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX) Dept. of Health and Human Services (US). Office of Evaluation and Inspections: 1994 Oct. Report No: HHSI-GOE 169200860.

Tez: Kaplan SI. Post-hospital home health care: the elderly access and utilization (dissertation). St. Louis (MO): Washington Univ. 1995.

Yayına kabul edilmiş ancak henüz basılmamış yazılar: Leshner AI. Molecular mechanisms of cocaine addiction. N Engl J Med In press 1997.

Erken Çevrimiçi Yayın: Aksu HU, Ertürk M, Gül M, Uslu N. Successful treatment of a patient with pulmonary embolism and biatrial thrombus. Anadolu Kardiyol Derg 2012 Dec 26. doi: 10.5152/akd.2013.062. [Epub ahead of print]

Elektronik formatta yayınlanan yazı: 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.

REVİZYONLAR

Yazarlar makalelerinin revizyon dosyalarını gönderirken, ana metin üzerinde yaptıkları değişiklikleri işaretlemeli, ek olarak, hakemler tarafından öne sürülen önerilerle ilgili notlarını "Hakemlere Cevap" dosyasında göndermelidir. Hakemlere Cevap dosyasında her hakemin yorumunun ardından yazarın cevabı gelmeli ve değişikliklerin yapıldığı satır numaraları da ayrıca belirtilmelidir. Revize makaleler karar mektubunu takip eden 30 gün içerisinde dergiye gönderilmelidir. Makalenin revize versiyonu belirtilen süre içerisinde yüklenmezse, revizyon seçeneği iptal olabilir. Yazarların revizyon için ek süreye ihtiyaç duymaları durumunda uzatma taleplerini ilk 30 gün sona ermeden dergiye iletmeleri gerekmektedir.

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