RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

DOI: 10.4274/atfm.galenos.2025.98624
J Ankara Univ Fac Med 2025;78(1):49-54

TEMEL TIP BİLİMLERİ / BASIC MEDICAL SCIENCES

Hypoxia Induced Downregulation of Na^+/H^+ Exchanger-1 Activity Decreases Tumor Cell Proliferation

Hipoksi ile Indüklenen Na^+/H^+ Değiş-Tokuşcusu-1 Aktivitesindeki Azalma Tümör Hücre Proliferasyonunu Yavaşlatıyor

Gül Şimşek, Hilmi Burak Kandilci

Ankara University Faculty of Medicine, Department of Biophysics, Ankara, Türkiye

Abstract

Objectives: Hypoxia and acidosis are the hallmarks of proliferative tumor microenvironment which can modulate the expression and function of Na^+/H^+ exchanger-1 (NHE1) via hypoxia inducible factor 1 (Hif). Here, we investigate the severity and time dependent effects of chronic hypoxia on NHE1 activity and its correlation with cell proliferation in mouse atrium tumor derived HL-1 cells.

Materials and Methods: NHE1 activity was recorded using intracellular pH (pH_i) sensitive dye cSNARF-1 (Leica SP5). Cell proliferation was assessed by live cell movie analyzer (Nanoentek, JuLI Br®) or immunofluorescence method.

Results: According to our results, mild chronic hypoxia (2% O_2, 48 hours) or shorter duration severe chronic hypoxia (1% O_2, 24 hours) did not affect cell proliferation and NHE1 activity. In contrast, long term dimethyloxalylglycine (DMOG, Hif stabilizer) or zoniporide (NHE1 inhibitor) incubations (21% O_2, 24/48 hours) suppressed cell proliferation.

Conclusion: When our published and current results in this study interpreted together, at a critical level and duration of chronic hypoxia, Hif mediated downregulation of NHE1 activity could suppress tumor cell proliferation regardless of the well-known anti-proliferative early term direct effects of Hif. Therefore, restriction of NHE1 activity in tumor hypoxia is an important alternative target in regulating anti-proliferative action against tumor cells.

Keywords: Cell culture techniques, hypoxia, sodium-hydrogen exchangers, neoplasms

Öz

Amaç: Proliferatif tümör mikroçevresinin başlıca özelliklerinden olan hipoksi ve asidoz, Na^+/H^+ değiş-tokuşcusunun (NHE1) ekspresyon ve fonksiyonunu hipoksi ile indüklenen faktör 1 (Hif) aracılığı ile etkileyebilmektedir. Bu çalışmada, fare atrium kökenli HL-1 tümör hücrelerinde farklı süre ve şiddetlerdeki kronik hipoksinin NHE1 aktivitesi üzerindeki etkisi ve hücre proliferasyonu ile ilişkisi araştırıldı.

Gereç ve Yöntem: NHE1 aktivitesi kaydı için hücre içi pH (pH_i) duyarlı boya cSNARF-1 (Leica SP5) kullanıldı. Hücre proliferasyonu canlı hücre görüntüleme sistemi (Nanoentek, JuLI Br®) veya immünofloresan yöntemleri ile ölçüldü.

Bulgular: Bu çalışmadaki bulgularımıza göre kronik hipoksi (%2 O_2, 48 saat) veya kısa süreli şiddetli kronik hipoksi (%1 O_2, 24 saat), hücre proliferasyonu veya NHE1 aktivitesini etkilemedi. Ancak, hücrelerin dimetiloksalilglisin (DMOG, Hif birikimine yol açar) veya zoniporid (NHE1 inhibitörü) ile normokside uzun süreli inkübasyonları (%21 O_2, 24/48 saat) hücre proliferasyonunu baskıladı.

Sonuç: Daha önce elde ettiğimiz bulgular ve bu çalışmadan elde edilen sonuçlar birlikte yorumlandığında, kritik şiddet ve süredeki kronik hipokside Hif aracılı NHE1 aktivitesindeki azalma, Hif'in iyi bilinen erken dönem anti-proliferatif etkilerinden bağımsız olarak tümör hücre proliferasyonunu azaltabilir. Dolayısıyla, tümör hipoksisinde NHE1 aktivitesini sınırlandırmak tümör gelişimine karşı etki hedeflenmesinde alternatif bir yaklaşım olarak önemlidir.

Anahtar Kelimeler: Hücre kültürü teknikleri, hipoksi, sodyum-hidrojen değiş-tokuşcusu, kanser

Address for Correspondence/Yazışma Adresi: Prof. Hilmi Burak Kandilci
Ankara University Faculty of Medicine, Department of Biophysics, Ankara, Türkiye
E-mail: kandilci@medicine.ankara.edu.tr **ORCID ID:** orcid.org/0000-0003-3050-6443

Received/Geliş Tarihi: 24.12.2024 **Accepted/Kabul Tarihi:** 06.02.2025 **Publication Date/Yayınlanma Tarihi:** 21.03.2025

Cite this article as/Atıf: Şimşek G, Kandilci HB. Hypoxia induced downregulation of Na^+/H^+ exchanger-1 activity decreases tumor cell proliferation. J Ankara Univ Fac Med. 2025;78(1):49-54

Introduction

Cell metabolism generates hydrogen ion (H^+) which is extruded out from the cytoplasm through intracellular pH (pH_i) regulatory proteins. Sodium hydrogen exchanger (NHE1) is a ubiquitously expressed integral membrane protein that regulates pH_i by removing a proton in exchange for an extracellular Na^+ ion (1). NHE1 is required for physiological cell proliferation as well as for tumor growth (2,3).

Hypoxic areas are frequently encountered in solid tumors due to decreased vascular growth and restricted oxygen diffusion (4,5). Oxygen deprivation stimulates hypoxia-induced factor 1 (Hif) accumulation in the cell which typically, in the presence of oxygen, is rapidly degraded by prolyl hydroxylase (PHD) enzyme. PHD enzyme is a 2-oxoglutarate-dependent dioxygenase which has been reported to be inhibited by 2-oxoglutarate analogue dimethyloxalylglycine (DMOG) leading to Hif accumulation even under normoxic conditions (6). Hif is known to affect cell proliferation at transcriptional level through regulation of cell cycle (7-10). Apart from these well-known mechanisms, Hif has also been reported to reduce DNA replication and suppress cell proliferation also through non-transcriptional pathways (11,12). Conversely, for cancer cells, Hif may contribute to increased cell growth (13).

Our aim was to investigate NHE1's role in cancer cell proliferation in chronic hypoxia. The methodology used in this study enabled us to maintain the extracellular pH constant throughout the chronic hypoxic period (acidic extracellular pH shift was prevented due to anaerobic respiration), which might be a factor itself in favoring tumor survival thereby it was eliminated (14). Moreover, we applied various hypoxia protocols that allowed us to dissect out the individual roles of Hif and its downstream target NHE1 activity in cell proliferation during chronic hypoxia. Our findings suggests that the Hif's established early term cell cycle suppressive effects might not be the major mechanism observed here. More likely, Hif suppresses cell proliferation mainly via regulating NHE1 activity at a critical hypoxic threshold. In addition, PHD and/or NHE1 inhibitors have direct effects in cardiac tumor cell proliferation. All these results point to new therapeutic targets for tumor tissue already proliferating in hypoxic environment.

Materials and Methods

Cell Culture and Induction of Hypoxia

Mouse atrial tumor-derived HL-1 cells were cultured as described previously (15). Incubation was performed under standard culture conditions (5% CO_2, 95% humidity, 37 °C) for 24-36 hours until 40-50% confluence. Grown cells were transferred to 60 mm petri dishes (two round glasses per petri dish) or to 6 wells (two fractured coverslip per well) containing fresh medium for proliferation assessment or functional experiments respectively. This maneuver increased the amount of medium per cell without changing gas diffusion parameters. Cells were then either incubated at 21% O_2 (normoxia control group) or at 1-2% (continuous hypoxia group) in an oxygen-controlled carbon dioxide incubator (Panasonic, MCO-170M-PE) for 24/48 hours. In standard culture conditions, hypoxic incubation reduces medium pH in 48 hours. By increasing buffering capacity (i.e., smaller cell density in larger volumes) medium acidification was prevented (i.e., medium pH was nearly kept constant at ~7.4) at the end of 24/48 hours of continuous normoxia/hypoxia incubation. Hypoxia mimetic agent, DMOG (1 mM) or NHE1 specific inhibitor, zoniporide (30 µM) was added directly into the medium and incubated in normoxia for 24/48 hours (16).

Superfusion of Cells and Confocal Microscopy

The immediate environment of cells was controlled by a superfusion system that provided rapid solution exchange. Solutions were delivered at 37 °C at 2 mL/min to a Perspex chamber with a coverslip bottom. Normal Tyrode solution (NT) contained 135 mM NaCl, 20 mM Hepes [4-(2-hydroxyethyl)-1 piperazine ethanesulfonic acid, pKa: 7,5], 11 mM glucose, 4.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$. In ammonium containing solutions, NaCl was isosmotically replaced with NH_4Cl. For pH_i imaging, cells were loaded for 30 min with the pH sensitive dye SNARF (Invitrogen, 5 µM) under standard culture conditions. The dye was excited at 543 nm in a confocal microscope (TCS SP5, Leica) and the emission signal was measured at wavelengths of 580 nm and 640 nm. The ratio was converted to pH_i using a calibration curve determined by the *in situ* nigericin (K^+/H^+ ionophore, 10 µM, Sigma) technique. Cells were acid loaded by ammonium prepulse method (rapid removal of 20 mM ammonium chloride superfusion, 4-5 min). NHE1 mediated H^+-extrusion (activity) was calculated as the product of the slope of pH_i recovery (dpH_i/dt) and intrinsic buffering capacity (β_i) in NT solutions. β_i were measured separately by gradual ammonium removal and data were fitted to a previously described model (16-18).

Live or Immunofluorescence Imaging of Cell Proliferation

Live Cell Movie Analyzer and software (JuLi Br, NanoEnTek Inc.) were used for instant measurement of cell proliferation during hypoxia (19). The device was placed in the culture incubator and real-time images were collected at 10 min intervals for 48 hours. Under these conditions, the lowest O_2 level attainable was stabilized at 2%. For more severe levels of hypoxia (1% O_2, 24/48 hours), device and cable connections were removed from the incubator as to latch the inside glass door properly therefore atmospheric O_2 contamination was

minimized. In this group, cell proliferation (i.e., the total number of cells) was visualized by the nuclear stain Yo-Pro-1 using immunofluorescence method as previously described (16). Zoniporide (30 µM) and DMOG (1 mM) were added to the cell medium at normoxia to observe the effect of direct inhibition of NHE1 activity or Hif on live cell proliferation.

Statistical Analysis

The number "n" in the experiments represents NHE1 activity or proliferation measured from the cell cluster grown on round or fractured coverslips. Two-way ANOVA test (GraphPad Prism 4.0) was used for the significant effect of hypoxia, drugs and its relationship with pH_i sensitivity (i.e., a change in pH_i sensitivity) in pH_i -NHE1 activity data sets obtained from experimental groups and controls. The NHE1 activity curves were fitted to a four-parameter logistic equation curve *(fit)*. In proliferation experiments, results were presented as mean ± standard error of the mean. Two-way Student's t-test was used for the difference between groups. $p<0.05$ was considered significant.

Ethics Committee Approval

The authors declared that this research does not require ethical approval. Since the study was not conducted on humans, consent was not obtained.

Results

Hif-mediated NHE1 Inhibition Decreases Tumor Cell Proliferation at a Critical Hypoxic Threshold

According to our previous findings, incubation of HL-1 cells in severe chronic hypoxia (1% O_2, 48 hours) or in DMOG (1 mM, 21% O_2, 48 hours) markedly reduced both NHE1 activity and cell proliferation (16). While in this study, NHE1 activity and cell proliferation was not affected when cells were incubated in severe chronic hypoxic conditions for shorter duration (1% O_2, 24 hours) or kept in milder chronic hypoxia (2% O_2, 48 hours) instead (Figures 1, 2). It is well documented that either chronic hypoxia (1-2% O_2, 24/48 hours) or DMOG incubation (1 mM, 21% O_2, 24/48 hours) triggers rapid accumulation of Hif in various cancer cell lines (16,20,21). Thus, we argue that, NHE1 activity and cell proliferation may not be altered even in the presence of Hif. On the other side, inhibition of NHE1 activity using NHE1 inhibitor (zoniporide, 30 µM) had effectively inhibited cell proliferation under normoxia (21% O_2, 24/48 hours) in a Hif independent manner (Figure 2A). Hence, these findings support the idea that, at a certain level and duration of hypoxia, Hif reduces cancer cell proliferation mainly through NHE1 inhibition. Hif's eminent anti-proliferative roles acting on cell cycle proteins at transcriptional and non-transcriptional levels which are usually detected at early stages of hypoxia seem to be of minor importance under these circumstances.

Prolyl Hydroxylase Inhibition Does Not Replicate the Effects of Hypoxia on Cell Proliferation

The PHD enzyme inhibitor DMOG is widely used to mimic the effects of hypoxia under normoxic conditions (22,23). However, our findings revealed that the effects of hypoxia and DMOG incubation on cell proliferation may be dissimilar. NHE1 activity and associated cell proliferation did not change in hypoxia (1% O_2, 24 hours), whereas DMOG incubation for the same period in normoxia had profound effect on cell proliferation (Figures 1, 2).

Discussion

Hypoxia is a condition in which the oxygen concentration in organs or cells is below the normal physiological level. Hypoxia is known to modulate normal and cancer cell proliferation (24). Particularly in solid tumors, rapid proliferation leads to tumor hypoxia, increases the risk of invagination and metastasis, decreases the efficiency of treatment by suppressing anti-tumor immunity (25). Interestingly, even in the presence of elevated Hif levels, cancer cells maintain proliferation (26). Accordingly, using basic research, it is important to reveal tumor specific hypoxic response mechanisms as to develop better targeted treatment approaches against tumor growth. However, reproducibility rate of preclinical experimental data in cancer research by another's is thought to be as low as 10% (27). The inadequate quality of the preclinical data obtained has been associated with the high failure rate (~95%) of newly developed compound transition from *in vitro* validation to phase 3 testing in cancer drug research (28). To improve data reproducibility, experimental control conditions must be

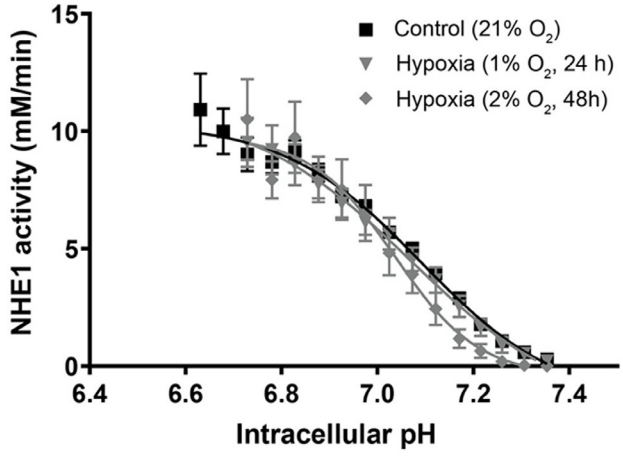

Figure 1: Intracellular pH (pH_i) sensitivity of NHE1 activity. NHE1 activity is pH_i dependent and increases at acidic pH_i. 48 h of mild hypoxia (2% O_2, ♦) or 24 h severe hypoxia (1% O_2, ▼) did not affect pHi sensitivity of NHE1 activity compared to control (■) (n: 19-24, $p>0.05$)

strictly controlled. One of the poorly controlled, fundamental variables is pH of the culture medium and biological processes are extremely sensitive to extracellular acid or base alterations. For example, extracellular pH alone is known to be effective on cell proliferation (29). Controlling medium pH in culture systems under continuous prolonged hypoxia is even more difficult because lactic acid produced as a result of increased glycolytic metabolism generally shifts medium pH to further acidic. We conducted empirical methodology in which medium buffering capacity was increased to prevent medium acidification so that single effect of hypoxia on proliferation would be observed. Continuous monitoring of the cells in hypoxia (2% O_2) for 48 hours allowed live monitoring of proliferation rate and was preferred to indirect proliferation measurement methods (e.g., ATP, MTT measurement). However, due to technical limitations (see methods section), proliferation in more severe hypoxic (1% O_2) conditions was measured using fixed preparations at the end of 48 hours hypoxic exposure (16).

Chronic hypoxia is known to affect cell proliferation through Hif dependent or independent mechanisms (30,31). In addition, Hif dependent NHE1 regulation has been shown in pulmonary artery (32), in HL-1 cells (16) and in various cancer cell lines (20). Furthermore, NHE1 protein also plays an essential role in cell proliferation through Hif independent pathway (33,34). Under these complex interactions, the aim of this study was to elucidate the unique role of NHE1 activity in cancer cell proliferation during chronic hypoxia. For this purpose, we tested the effects of duration of mild chronic hypoxia, and duration of severe chronic hypoxia on NHE1 activity and proliferation. Firstly, Hif's role in cell proliferation was dissected. Our findings and other studies (21) in HL-1 cells demonstrated that 24 hours of hypoxia (1% O_2 or 2% O_2) induces Hif accumulation where cell proliferation was not affected. These results initially indicate that, anti-proliferative effects may not be apparent in 24 hours. However, cell proliferation also did not change at prolonged exposure of mild hypoxia (2% O_2, 48 hours) (Figure 2A), possibly in the presence of the reported Hif accumulation under similar hypoxic conditions (20). Thus, in tumor derived HL-1 cells, Hif might not suppress proliferation. To mimic those effects, DMOG's effect was also tested. DMOG incubation at 24 hours was reported to induce Hif accumulation in HL-1 cells (21). Unexpectedly, DMOG incubation for 24 hours in normoxia did not mimic hypoxia results and decreased cell proliferation (Figure 2A). Accordingly, these results strengthen the idea that, DMOG does not provide an accurate replication of hypoxia and may decrease cell proliferation Hif-independently (35). DMOG is an inhibitor of 2-oxaglutarate-dependent dioxygenase enzymes which also may activate other Hif-independent signaling pathways (35,36). On the other hand, our previous findings clearly showed that proliferation decreased in a Hif dependent manner at severe chronic hypoxia (16). Therefore, we postulate that the critical hypoxia level and duration that declined cancer cell proliferation occurred only at 1% O_2 and in 48 hours. At this threshold level of hypoxia, we also previously assessed cell viability by measuring pH_i regulation as to preclude apoptotic cells under strong hypoxic stimuli (16). Nevertheless, these

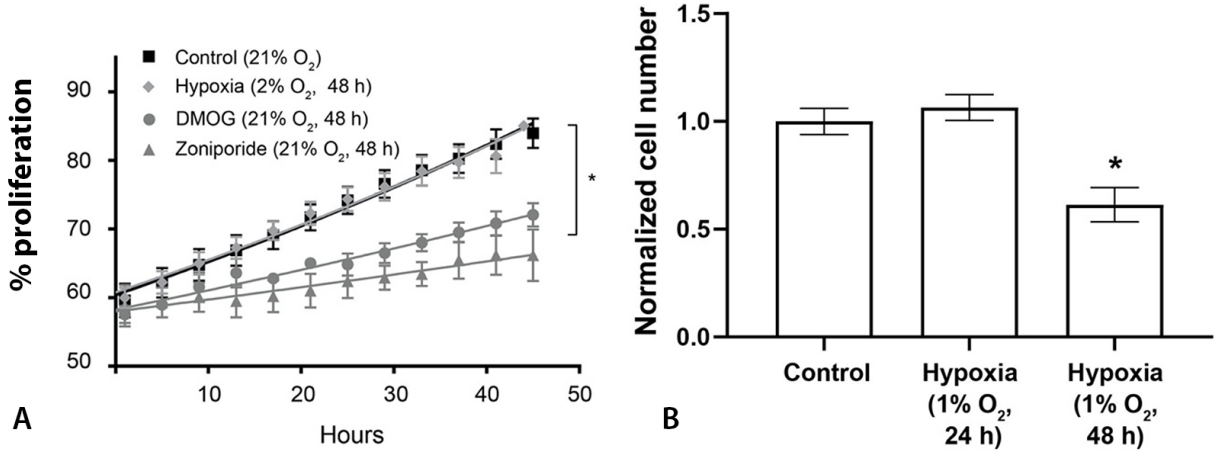

Figure 2: Image based live measurements of cell proliferation depending on confluence (area) detection or proliferation was assessed from cell counts, made from Yo-Pro-1 staining in fixed preparations. A) For live measurements, initial cell density was seeded to be ~60% confluence and then monitored in real time for 48 h in all experimental groups. At the end of the experiment, normoxic control cell confluence ■ was measured as ~80%. Incubation with DMOG (1 mM, •) or zoniporide (30 µM, ▲) for 24/48 h in normoxia decreased, whereas 2% O_2 hypoxia (♦) exposure did not alter cell proliferation. B) Due to technical limitations (see Materials and Methods), proliferation during severe hypoxia (1% O_2) were measured by immunofluorescence and cell numbers were counted from nuclei staining (2.5 µM, Yo-pro-1). Experimental groups were compared using the same confocal microscope settings (magnification) (n=3, *p<0.05, *compared to control). Note: 1% O_2 48 h data were adopted from (15) for comparison

results address at least in HL-1 cells that, Hif probably do not mainly alter cell-cycle progression directly through putative transcriptional targets where these effects were observed at the early stages (6-24 hours) of hypoxia (2% O_2) (37,38). Indeed, it appears more likely that Hif exerts influences on certain post translational pathways affecting proliferation in tumor cells. Our data emphasize the effect of hypoxia (i.e., Hif's) and NHE1 activity on cell proliferation is correlated. In other words, in the presence of Hif accumulation, 24 hours severe hypoxia doesn't alter NHE1 activity or proliferation whereas 48 hours severe hypoxia/DMOG incubation inhibits both NHE1 activity and proliferation. Thus, we propose the main effect of Hif on tumor proliferation is mediated through NHE1 activity in chronic hypoxia.

To support this, NHE1's effect on proliferation is further investigated by 48 hours zoniporide incubation in normoxia. Zoniporide incubation blocked cell proliferation at 24/48 hours. Our preliminary findings predicts that NHE1 inhibition did not necessarily disturb pH_i homeostasis in the presence of HCO_3^- ion. That is, acute zoniporide application did not alter basal pH_i showing that it's pH_i regulatory role could be compensated by HCO_3^--mediated transport (data not shown). This preliminary data makes it difficult to explain its role in cell proliferation due to alkaline shift of the steady state pH_i, induced by the blockage of NHE1 activity (2,3). Correspondingly, the pyrazinguanidine-based chemical structure of the NHE1 inhibitor zoniporide used in this study has been reported to reduce cell proliferation independently from NHE1 inhibition (39). Hence, the exact mechanism of NHE1 protein's antiproliferative action remains to be elucidated. But, NHE1 inhibitors are unlikely to inhibit PHD thereby leads to Hif accumulation in normoxia (40). As a result, we do not rule out the nonspecific actions of zoniporide or DMOG on cell proliferation, but the correlation between NHE1 regulation and cell proliferation in hypoxia still points to the importance of Hif regulated NHE1 activity. The findings of this current study and the related literature are summarized in Table 1.

Table 1: Summarized results and review of the related literature

	Hypoxia (%1 O_2)		Hypoxia (2% O_2)		DMOG (%21 O_2)		Zoniporide (%21 O_2)	
	24s	48s	24s	48s	24s	48s	24s	48s
Hif accumulation	↑*		↑#	↑**	↑#	↑*		
NHE1 activity	↔	↓*		↔		↓	↓	
Proliferation	↔	↓	↔		↓		↓	

Note: (↔) unchanged (↑) increased (↓) decreased compared to control. * The data are from (15), ** The data are from (19), # The data are from (20)

Conclusions

In summary, we propose that Hif downregulates NHE1 activity which leads to decrease in tumor cell proliferation. This relationship implies restriction of NHE1 activity as a key candidate for anti-proliferative action. Additionally, considering that NHE1 is internalized from membrane under severe chronic hypoxic conditions, NHE1 may also be an alternative target for drug delivery against tumor cells. Apart from this mechanism, inhibition of NHE1 activity with pyrazinguanidine-based compounds (such as zoniporide, amiloride) have additional anti-proliferative effects, overall suggesting them as promising compounds in cancer treatment.

Ethics

Ethics Committee Approval: The authors declared that this research does not require ethical approval.

Informed Consent: Since the study was not conducted on humans, consent was not obtained.

Footnotes

Authorship Contributions

Concept: H.B.K., Design: H.B.K., Data Collection and/or Processing: G.Ş., Analysis and/or Interpretation: G.Ş., H.B.K., Literature Search: G.Ş., H.B.K., Writing: H.B.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: This work was supported by Ankara University Scientific Research Project Unit Grant Number: TSG-2022-2410.

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