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CONTENTS

EXPERIMED

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ORİJİNAL ARAŞTIRMA

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Signal Crosstalk Promoted Proliferative Lesions in Mouse Mammary Glands As a Consequence of ET-1 Overexpression

ET-1 Aşırı Anlatımının Bir Sonucu Olan, Sinyal Çapraz Etkileşimi 'Crosstalk', Fare Meme Bezlerinde Proliferatif Lezyonların Oluşumunu Teşvik Eder

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ABSTRACT

Objective: As a distinct cellular signaling model, a receptor crosstalk between *G protein-coupled receptors (GPCR)* and *epidermal growth factor receptor (EGFR)* has been demonstrated in various *in vitro* studies. In addition, recent *in vitro* studies had focused on the signaling pathways of *endothelin-1 (ET-1)* in the pathophysiology of cancer. Accordingly, a growing interest in the analysis of the receptor crosstalk between *ET-1* receptors and EGFR and functional consequences of EGFR activation of proliferative diseases evoked us to the analysis of this phenomenon *in vivo*.

Materials and Methods: We performed a comparative study between *ET-1* transgenic mice and control mice during the late pregnancy (n=7), early lactation (n=6) the mid of lactation (n=10) and involution day 14 (n=7) periods. Hematoxylin and eosin (HE)-stained parallel sections from mammary glands were microscopically examined. The key signal proteins (*ETAR, ETBR, ERK1/2, pEGFR*) in transactivation of *EGFR* were analyzed employing Western blot techniques. Genes (*amphiregulin, TGFa, EGF, HB-EGF, ADAM 17*) known to play an important role in these activities were analyzed using real-time PCR (RT-PCR) techniques.

Results: *ET-1* transgenic mice exhibited hyperproliferative lesions (*lactational hyperplasia*) during the middle of the lactation period. Our RT-PCR analyses showed a prominent up regulation of *amphiregulin* and *ADAM 17* in *ET-1* transgenic mice. Moreover, we found higher *EGFR* and *ERKs* activations in the transgenic mammary glands.

Conclusion: This study highlights a causative effect of upregulated *ET-1* gene expression on the induction of proliferative lesions via *EGFR* transactivation in mammary glands. Further, *ET-1* overexpression induced an upregulation of *amphiregulin* and *ADAM17* expressions in the transgenic mammary glands. These results suggests that the enhanced *ET-1* gene expression and its receptors might have a crucial role in proliferative diseases maintaining *EGFR* activation.

Keywords: *Endothelin-1*, *EGFR* transactivation, lactational hyperplasia, *amphiregulin*, transgenic mice

ÖZ

Amaç: Farklı bir hücresel sinyalleşme modeli olarak, *G proteini ile eşleşmiş reseptörler (GPCR)* ve *epidermal growth faktör reseptör (EGFR)* arasındaki reseptör çapraz etkileşimi (crosstalk), çeşitli *in vitro* çalışmalarda gösterilmiştir. Ayrıca, son dönem *in vitro* çalışmalar, *endotelin-1 (ET-1)* sinyal yolağının kanser patofizyolojisi üzerindeki ilişkisine odaklanmıştır. Bu bağlamda, *ET-1* reseptörleri ve *EGFR* arasındaki reseptör çapraz etkileşim analizine ve *EGFR* aktivasyonunun fonksiyonel sonuçlarından proliferatif hastalıklara olan artan ilgi, bize bu fenomeni *in vivo* analiz etme fikrini oluşturmuştur.

Gereç ve Yöntem: *ET-1* transgenik fareler ve kontrol fareleri arasında geç gebelik dönemi (n=7), erken laktasyon dönemi (n=6) orta dönem laktasyon (n=10) ve involusyon 14. gün (n=7) sırasında karşılaştırmalı bir çalışma gerçekleştirildi. Meme bezlerinden hematoksilen-eozin (HE) ile boyanmış paralel kesitler mikroskobik olarak incelendi. EGFR'nin transaktivasyonunda görev alan anahtar sinyal proteinleri (*ETAR, ETBR, ERK1/2, pEGFR*), Western blot teknikleri kullanılarak analiz edildi. Bu aktivitelerde önemli bir rol oynadığı bilinen genler (*amfiregulin, TGFa, EGF, HB-EGF, ADAM 17*), gercek zamanlı polimeraz zincir reaksiyonu (RT-PCR) teknikleri kullanılarak analiz edildi.

Bulgular: *ET-1* transgenik fareler, laktasyon döneminin ortasında hiper-proliferatif lezyonlar (*laktasyonel hiperplazi*) geliştirdi. *RT-PCR* analizlerimiz, transgenik farelerde *amfiregulin* ve *ADAM 17* gen anlatımlarında belirgin bir artış olduğunu göstermektedir. Ayrıca transgenik meme bezlerinde daha yüksek *EGFR* ve *ERKs* aktivasyonları tespit edilmiştir.

Sonuç: Bu çalışma, yüksek seviyede *ET-1* gen anlatımının *EGFR* yi transaktive etme yoluyla meme bezlerinde proliferatif lezyonları tetiklediğini göstermektedir. Ayrıca, *ET-1* aşırı anlatımı, transgen meme bezlerinde *amfiregulin* ve *ADAM17* anlatımlarının belirgin bir şekilde yukarı regülasyonuna neden olmaktadır. Bu sonuçlar bize *ET-1* gen ekspresyonunu ve reseptörlerinin *EGFR* aktivasyonunu sağlayarak proliferatif hastalıklarda önemli bir role sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Endotelin-1*, *EGFR* transaktivasyonu, laktasyonel hiperplazi, *amfiregulin*, transgenik fareler

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INTRODUCTION

Endothelin-1 (ET-1), an important substance in the maintenance of vascular tone, was firstly isolated and characterised by Yanagisawa from cultured supernatant of porcine aortic endothelial cells (6). *ET-1* is expressed as an inactive precursor peptide comprising of 212 amino acid residues, named preproendothelin. Two endopeptidases control the activation process of preproendohelin (1). Firstly, the prepro form of endothelin is cleaved by a dibasic endopeptidase to form big *ET-1* or *proET-1* which is biologically inactive. Secondly, the conversion of *proET-1* to the biologically active *ET-1* is catalysed by a furin like protease named *endothelin converting enzyme (ECE)*. The cleavage occurs in the intracellular compartments and on the cell surfaces and leads to the active *ET-1*. The active *ET-1* comprises 21 amino acids and is folded by two disulphide bridges designating the half-life of the biological activity (2). The half-life of *ET-1 in vivo* is less than one minute whereas *proET-1* lasts approx. 20-25 mins (3). Therefore, under normal physiological conditions *ET-1* is not a circulating hormone but rather *ET-1* functions in an autocrine or paracrine manner at multiple locations in the body (4,5).

ET-1 exerts its effect by binding to two distinct *G protein-coupled receptors (GPCR)*, the *endothelin A (ETAR) and endothelin B receptor (ETBR)*. However, on the functional level both receptors differ, *ETAR* predominantly mediates vasoconstriction in vascular smooth muscle cells (6) whereas, *ETBR* activation results in vasodilatation in vascular endothelial cells (7).

In addition to typical *GPCR* signal characteristics, these receptors are also able to communicate with structurally unrelated receptors (transactivation) such as the epidermal growth factor receptor (*EGFR*), the most prominent receptor tyrosine kinase, (8-10) resulting in *EGFR* characteristic intracellular signals (Figure 1). The *EGFR* mediated signaling pathway is positioned to affect duct formation as well as the outgrowth and branching of the mammary gland during pregnancy (11-13).

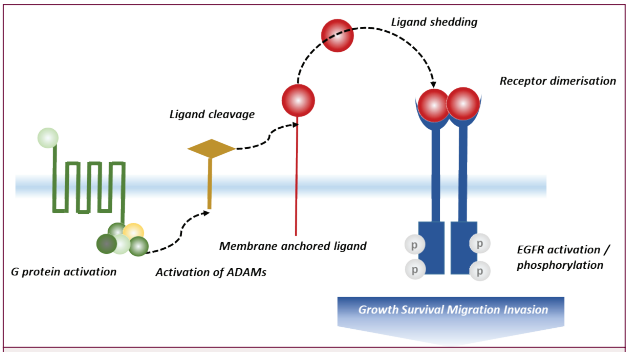

Figure 1. *EGFR* Transactivation mechanism: G Protein Coupled Receptor (*ETAR / ETBR)* activates metalloproteases *(ADAM 17*) which cleave and release the inactive *EGFR* ligands (*amphiregulin*) from cell surface. Free active ligand amphiregulin binds to its receptor (*EGFR*) and lead the downstream signaling cascade of *EGFR* (*illustrated by Nadir Gül*).

Further, recent studies reported that morphogenesis of the mammary gland requires paracrine activation of the *EGFR* via metalloprotease dependent shedding of *amphiregulin* (14,15). Transactivation of the *EGFR* is regulated by various cellular responses such as overexpression, amplification or mutation of critical pathway elements with variable functional outcomes which are frequently linked to hyperproliferative diseases.

Indeed, *GPCR* induced *EGFR* transactivation was found to mediate cell proliferation in breast cancer cells (16,17) and an increase of tumorigenicity in ovarian cancer cells (18). Activation of *EGFR* affects a wide range of cellular responses, depending on the coordinate expression of the cognate ligand (19). Up to now, eight EGF like ligands that directly activate the *EGFR* have been identified: *EGF, TGFα, heparin binding-EGF (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen and cripto* (20-25). Each of these molecules activates receptors of the HER family of tyrosine kinase receptor by autocrine or paracrine stimulation (26). The upregulation of these ligands is believed to be critical for the tumor growth.

A number of studies point out receptor crosstalk under controlled experimental models such as cancer cell lines. The purpose of this study was to compare the results of the above-mentioned *in vitro* studies under *in vivo* and non neoplastic physiology. Therefore, we also systematically analyzed possible tumorigenic effect of *ET-1* via receptor transactivation on *ET-1* overexpressing mice model.

MATERIALS AND METHODS

Mouse Models

In this study the outbred strains NMRI (Harlan-Winkelmann, Paderborn) as wild type and NMRI originated homozygotic human ET-1 transgenic mice were used. These transgenic mice have been extensively characterized for their cardiovascular and renal phenotypes (27).

The animal experiments were maintained by dividing the groups of animals under three physiologic conditions; Pregnancy day 18 (n=7), lactation day 3 (n=6), lactation day 14 (n=10) and involution day 14 (n=7).

Mammary gland samples were obtained free of the muscles of the anterior abdominal wall and skin by blunt dissection on late pregnancy and lactation physiological stages. Animals had free access to standard mouse chow (mouse chow 5015) and tap water. The mice were routinely screened for common mouse pathogens.

The animals were sacrificed by cervical dislocation. In general, male and female mice were held separately in groups of 2-6 animals. All animal experiments were conducted in accordance with the German Law for animal protection (Tierschutzgesetz).

Histology, Immunohistochemistry; The mammary glands were fixed in paraformaldehyde for a minimum of 12 hours at room temperature. After the fixation process, the tissue sam-

ples were embedded in paraffin blocks and cut in at 5mm sections. The sections were submitted to hematoxylin–Eosin (HE) staining or incubated with SMA antibody. These slides were evaluated with blinded analytical protocol.

Western Blots Analyses

Frozen mammary gland samples were pulverized in LN2 with a mortar and a pestle. 100 mg tissue samples were homogenized in urea lysis buffer. After the centrifugation, the supernatants were placed into new tubes. Protein concentration was measured with the Bradford colorimetric assay.

Samples (25µg) were run on SDS (12%) polyacrylamide gels, blotted onto nitrocellulose membrane (Amersham) and incubated with blocking solution in TBS with 0.1% tween 20 for 1 hour at room temperature. Membranes were incubated with primary antibody diluted in blocking solution overnight at 4°C and detected using horseradish peroxidase conjugated secondary antibodies in conjunction with ECL (Amersham).

Immunoprecipitation; Grinded mammary gland samples were lysed in ice cold RIPA buffer. Lysates containing 400 µg of proteins in RIPA buffer were incubated with 2µg/ml *EGFR* antibody at 4°C overnight. The immune complex was precipitated with protein A sepharose CL-4B beads at 4°C for 1hr. The beads were washed (3 times) with PBS containing tween-20 (1%) and spin down by centrifugation (2000 rpm for 1 min), re-suspended in RIPA buffer and boiled for 5 min at 95°C in a thermomixer (Eppendorf). The samples were spin-down at 2000 rpm for 1 min. The supernatants were carefully placed in new tubes and immunoblotted with *EGFR (pY845)* and *EGFR (Y1005),* respectively (Table 1).

Gene Expression Analyses

Total RNA was extracted from quick-frozen mammary glands (50-100 mg per sample) using standard trizol extraction method (Invitrogen). RNA quantification was performed with a bio-analyzer (Agilent 2100) using a microfabricated chip (Agilent RNA 6000 Nano). The system was able to report not only the amount of RNA but also the 260/280 Nm ratio and the RNA integrity number (RIN). RIN is a scale (from 1 to 10) to measure degradation and quality of RNA. Higher RIN value represents a better quality of RNA. Less than 9 RIN scaled samples were not used.

Prior to cDNA synthesis, all crude RNA samples were routinely treated with DNase1 (TurboDNase, Ambion). cDNA was synthetized from 1000 ng of total RNA using Promega MMLV reverse transcriptase enzyme with oligo(dT)18 Primers according to the manufacturer's protocol.

For the quantitative detection, the primers were designed using Ensembl gene data base and employing a commercial software (Primer 3) and web based algorithms (NCBI Blast, multi align) otherwise, the primer sets used from the literature were specified. All these primers were purchased from Invitrogen, Oligo (dT)18 were obtained from Promega (Table 2). The quantitative PCRs were performed using sybergreen mastermix (Applied Biosystems) and were carried out (25 ng template per test) in triplicate in MX 3700p Real-time PCR (RT-PCR) system (Stratogene).

Statistical Analyses

Western blots analyses; The digitized membranes were evaluated by special software (AlphaEaseFC) and the densitometric data were analyzed using a student's t-test by a statistical analysis program (Graphpad prism).

Gene Expression Analyses; Primer efficiency, standard curve and expression analyses were performed with the software Relative Expression Software Tool (REST 2008) which is using Pair Wise Fixed Reallocation Randomisation Test for statistical analyses (28).

RESULTS

ET-1 acts through its two G-protein coupled receptors, the *ETAR* and the *ETBR*. To determine whether overexpression of *ET-1* in transgenic animals was accompanied by changes in the expression levels of these two receptors, expression of both re-

Table 1. The following antibodies were used in immunoprecipitation and Western blot analyses

Antibody	Origins and Dilutions	Molecular weight (kDa)	Reference
pEGFR	Rabbit polyclonal antibody (tyr 845)	170	Cell signaling Tech.
EGFR	Rabbit polyclonal antibody (tyr 1005)	170	Santa Cruz Biotech.
ETAR	Rabbit polyclonal antibody	69	Cell signaling Tech.
ETBR	Rabbit polyclonal antibody	49/34	Cell signaling Tech.
pERK	Rabbit polyclonal antibody	42/44	Cell signaling Tech.
ERK	Rabbit polyclonal antibody	42/44	Cell signaling Tech.
SMA	Mouse monoclonal antibody	--	Sigma
Antimouse Ig	Rabbit, HRP linked polyclonal antibody	--	Dakocytomation
Antirabbit Ig	Goat, HRP linked polyclonal antibody		Dakocytomation

Table 2. The following primer sets were used in RT-PCR analysis.

Gene	Forward Primers (5'-3')	Reverse Primers (5'-3')
ADAM 17 (mouse) (56)	ACTCTGAGGACAGTTAACCAAACC	AGTAAAAGGAGCCAATACCACAAG
Amphiregulin (mouse)	TCTTGGGCTTAATCACCTGTTC	GGGGACTACGACTACTCAGAG
EGF (mouse)	CTAAGGATCCTGACCCCGAACT	GTACAGCCGTGATTCTGAGTGG
EGFR (mouse)	GCCAATAATGTCTGCCACCT	TCCCAGTGGCAATAGATGGT
ETAR (mouse)	GCTGGTTCCCTCTTCACTTAAGC	TCATGGTTGCCAGGTTAATGC
ETBR (mouse)	TGTGCTCTAAGTATTGACAGATATCGAG	GGCTGTCTTGTAAAACTGCATGA
HB-EGF (mouse)	TGAACCTTTTCAAAGTTGCTTTCT	CGTGGATGCAGTAGTCCTTGTA
GAPDH (mouse)	CTTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGAG
TGFa (mouse)	CAGAGGGCAGTACAGTTGATTCAG	GAAGACATCCTGGGCAAGC

ceptors in the mouse mammary glands of day 3 lactating wild type and transgenic *ET-1* mice was determined at the mRNA and the protein level by RT-PCR and Western blotting, respectively. The mRNA expression of the *ETAR* and *ETBR* in *ET-1* transgenic mice did not differ from wild type animals. Additionally, this result was confirmed at the protein level for the two receptors (Figure 2).

Throughout the second half of lactation, some parts of the mammary gland displayed intense lobular proliferation with cells characterized by increased cytoplasmic volume and enlarged nuclei (Figure 3 C, D) in the transgenic group.

Although no definite signs of pathological dysplasia were detected, alveolar organization was lost in parts of these areas. Therefore, immunohistostaining of smooth muscle actin was employed in order to rule out an underlying malignant transformation. HE and *smooth muscle actin (SMA)* immunohistostaining was performed on parallel sections obtained from wild type and *ET-1* transgenic mammary glands. The slides were immunostained with an anti-*SMA* antibody and show brown colored myoepithelial cells enclosing the alveolar epithelial cells blue colored (Figure 3, F, H). The slides not treated with *SMA* antibody were utilized as negative control, ensuring specifity of the *SMA* immunohistostaining (Figure 3 E, G). *SMA* immunostaining clearly manifests the non-neoplastic structure by the presence of myoepithelial cells surrounding alveoli in the *ET-1* transgenic mice.

EGFR activation has an important role in mammary ductal outgrowth and branching (29,30). Deregulation of the activity of this receptor has a strong correlation with tumor progression

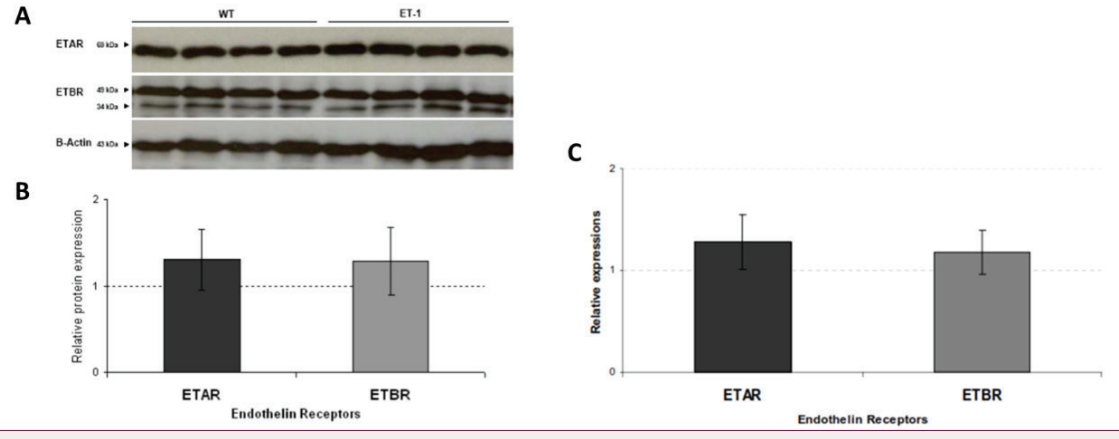

Figure 2. A. Western blot analysis of the two *ET-1* receptors *ETAR* (69kDa) and *ETBR* (49/34kDa) in mammary glands of wild type and transgenic mice. *Beta-actin* (43kDa) served as loading control. B. Densitometric analysis of endothelin receptors at lactation day 3, densities of the receptors were normalized with Beta-actin density n=4 females for each group. C. Analysis of the relative mRNA expression levels of the two *ET-1* receptors, *ETAR* and *ETBR*, in mammary glands at lactation day 3 employing RT-PCR. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). n=4 females for each group no significant difference was observed.

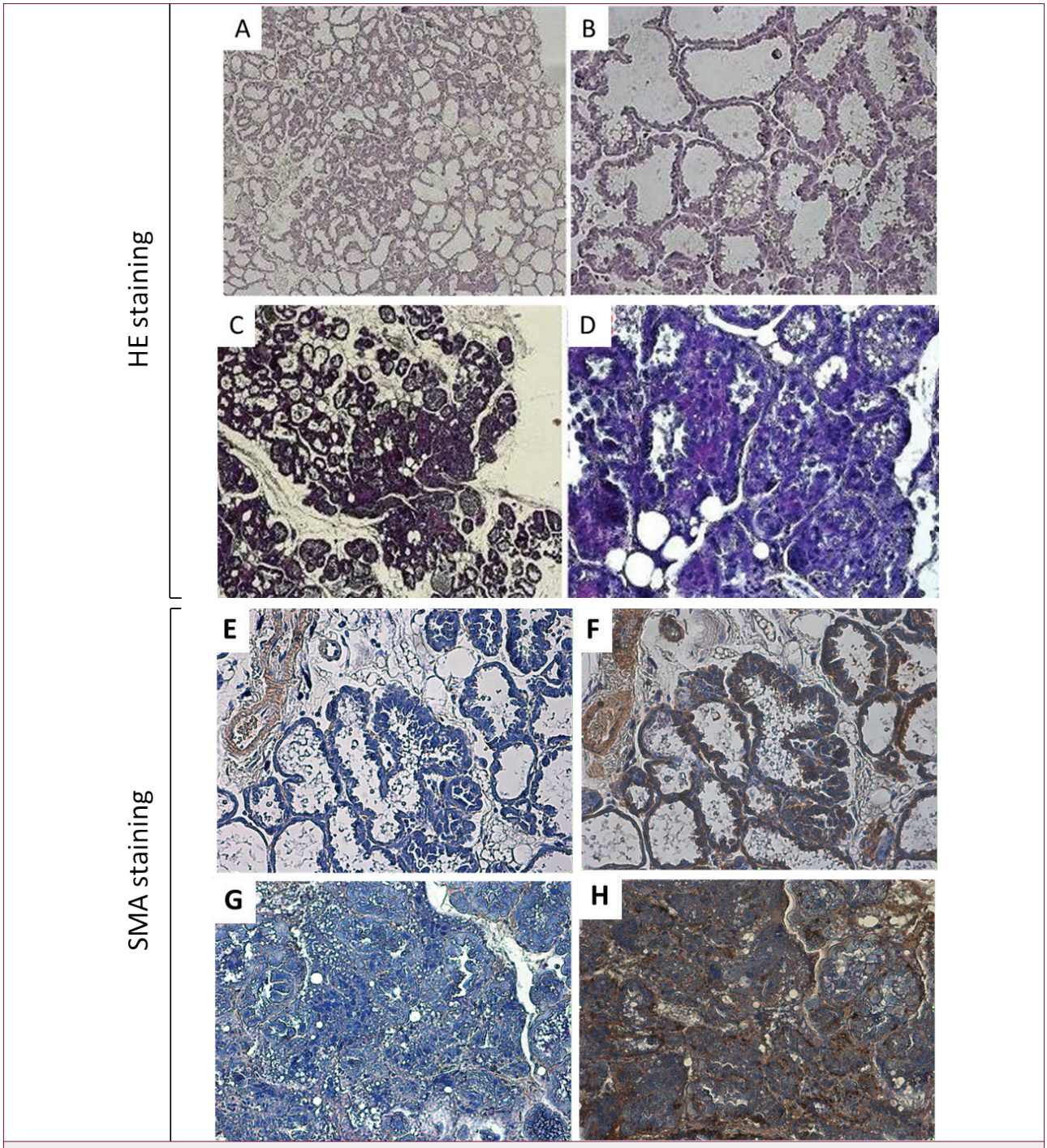

Figure 3. Histological features of the mammary gland at the lactation day 14. HE stained tissue sections; wild type: A; wild type: B; *ET-1*: C (x 50 magnification); *ET-1*: D (x 200 magnification). Wild type mammary glands exhibited fully expanded alveoli reflecting the highly active secretory capacity (A, B). *ET-1* transgenic mammary glands exhibited focal hyper proliferative lesions (C, D). SMA stained tissue sections; in order to visualize any possible cross reactivity for *SMA* staining parallel slides were treated without *SMA* antibody as negative control. Wild type: E; *ET-1*: G (x 200 magnification), myoepithelium was stained by a *SMA* specific immunohistostaining method; brown colored areas in both groups indicated the *SMA*, wild type: F; *ET-1*: H (x 200 magnification), the wild type group demonstrated well organized alveoli and the individual alveoli are surrounded by blood vessels. On the other hand, the *ET-1* group demonstrated hyper proliferative epithelium with *SMA* staining alveolar lumen and borders of alveoli become visual.

(31). Lactational hyperplasia (Figure 3 G, H) could be related to *EGFR* activation due to its central role on tumorigenesis. Therefore, *EGFR* phosphorylation was analyzed at lactation day 14 using immunoprecipitation techniques. As demonstrated in figures 5, an increased phosphorylation at residue *Y845* of the *EGFR* was observed in *ET-1* transgenic mice compared to wild type. The phosphorylation at residue *Y998* and *Y1068* of the *EGFR* did not changed (data is not shown).

*ERK*s represent critical downstream molecules for *ET-1* induced *EGFR* transactivation and might therefore be related to initiation of lactational hyperplasia. Hence, *ERK* activation was analyzed at pregnancy day 18 and lactation day 3 (Figure 4 C, D). The Western blot analysis represented that during pregnancy day 18, as well as during lactation day 3, a pronounced activation of *ERK* 1 and *ERK*2 signaling could be detected in the mammary glands of *ET-1* transgenic mice when compared to wild type mice (Figure 4E).

In order to identify ligands which are potentially involved in the observed activation of the *EGFR*, RT-PCR analyses were employed and the expression of *EGF, TGFa, HB-EGF* and *amphiregulin* was quantified at the transcript level.

As shown in figure 5A, expression analyses on the 14th day of lactation demonstrated only a significant increase for the *amphiregulin* level in *ET-1* transgenic compared to wild type mice, whereas the expression level of other ligands remained unchanged.

It is known that, *amphiregulin* expression was found to be increased during pregnancy but after parturition its transcription dramatically decreased due to its functional role on ductal development of the mammary gland (32,33). Whether this significant upregulation of *amphiregulin* synthesis caused by high *ET-1* expression, RNA levels of *amphiregulin* were analyzed at pregnancy day 18. As depicted in Figure 5B *amphiregulin* is also significantly upregulated at pregnancy day 18.

ADAM 17, the transmembrane metalloproteinase, is responsible for *amphiregulin* activation (54). Therefore, *ADAM 17*, as a key component of this signaling mechanism, was also analyzed employing RT-PCR techniques. As shown in figure 5C, *ADAM 17* expression was significantly upregulated in *ET-1* transgenic mice.

Following the lactation period, *ET-1* transgenic mice were analysed with HE staining in order to find out the presence of

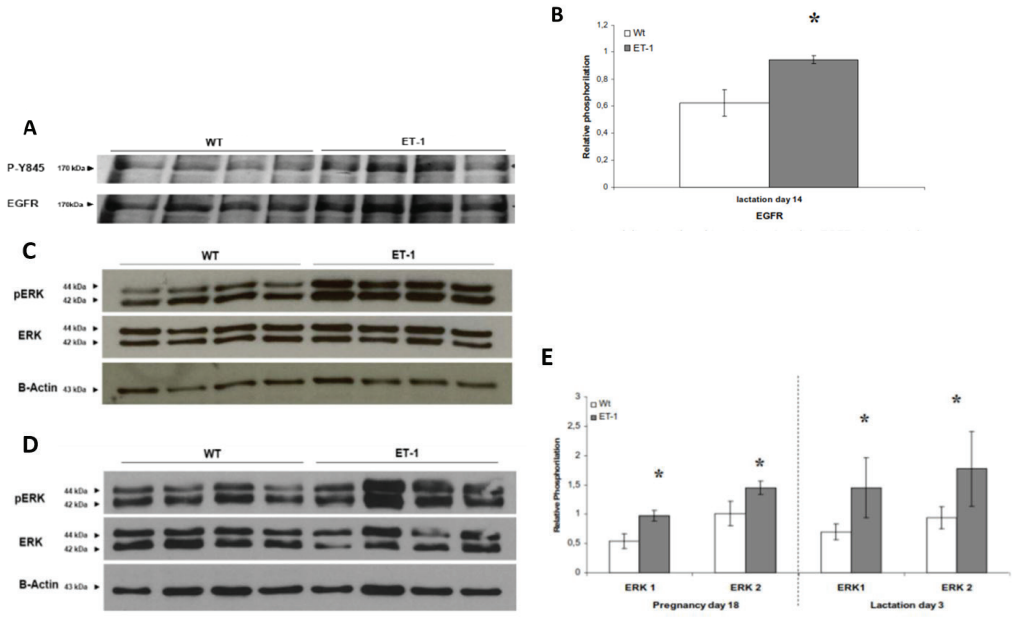

Figure 4. A. Analysis of the *EGFR* phosphorylation at lactation day 14 was determined by immunoprecipitation. ~400 µg protein lysate for each sample has been used for immunoprecipitation with an *EGFR* specific antibody (with *Y1005*), activation of *EGFR* has been monitored by phosphor specific antibody *Y845* and *EGFR* served as loading control. B. The representative densitometric analysis of relative *EGFR* phosphorylation in wild type and *ET-1* transgenic mice on the 14th day of lactation. *ET-1* transgenic mammary glands exhibited significantly more phosphorylated *EGFR* than the wild type counterparts C. At 18th day of pregnancy, protein expression and phosphorylation of *ERK* 1 (44kDa) and *ERK* 2 (42 kDa), proteins were determined by Western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. D. 3 rd. day of lactation protein expression and phosphorylation of *ERK* 1 (44kDa) and *ERK* 2 (42 kDa). E. The representative densitometric analysis of relative *ERK* 1 and *ERK* 2 phosphorylation *ET-1* transgenic mammary glands exhibited significantly more phosphorylated *ERK* 1 and *ERK* 2 than the wild type counterparts (n=4 mice for each group. (*) p<0.05 Student´s t-test).

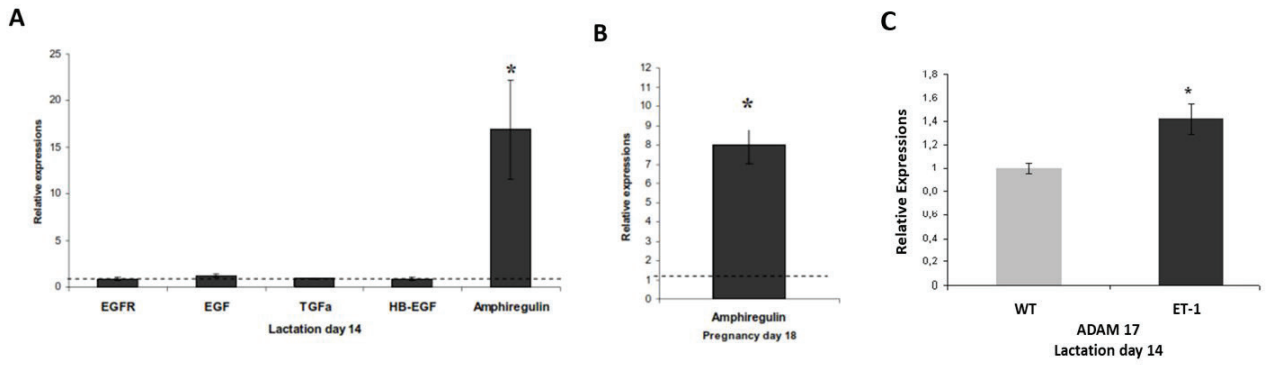

Figure 5. A. Relative expression of *EGFR* and its prominent ligands EGF, TGFa, HB-EGF, Amphiregulin at lactation day 14. B. Relative expression of *amphiregulin* at pregnancy day 18 C. Relative expression of ADAM17 at lactation day 14. The relative expression levels were compared to wild type mice expression levels, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (28) $p<0.05$ n=5 mice for each group.

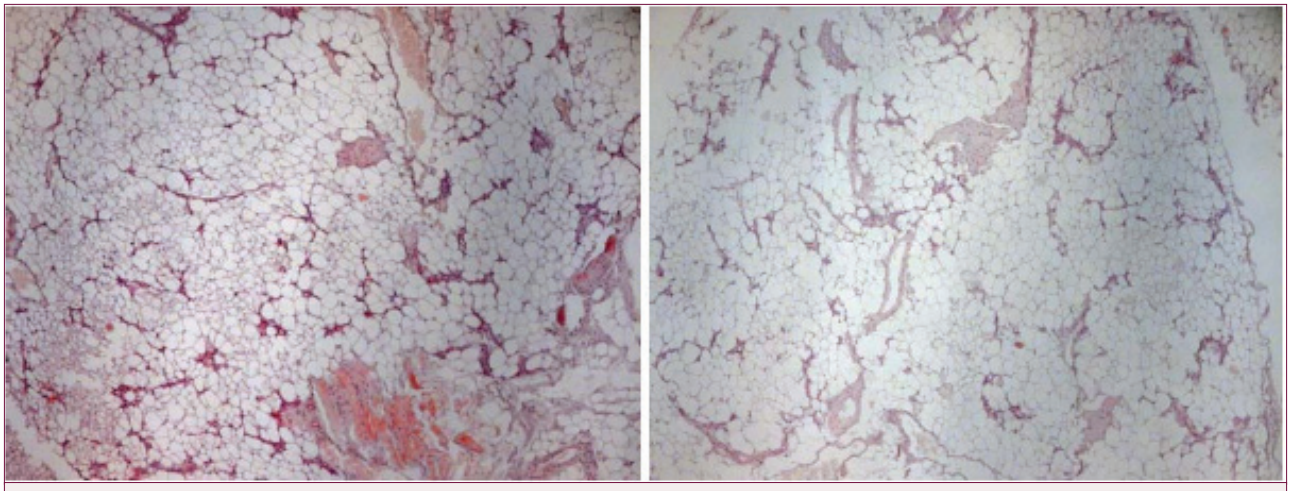

Figure 6. Histological features of the mammary gland at the 14th day of involution. The tissue sections were stained with HE of wild type (left) and *ET-1* transgenic mammary glands (right) (x 50 magnification). Both mammary glands showed same histological patterns of involution with the adipocytes and rudimentary epithelium. No hyperproliferative lesion or any type of irregularity was detected in both animal groups.

benign tumors at the involution day 14 stage. Throughout the involution no proliferative lesions were detected (Figure 6).

DISCUSSION

Along with its well-known effect on cardiovascular biology, *ET-1* has also been associated with a wide range of biological activities including mitogenic and proliferative responses in vascular smooth muscle cells and other cell types (34). However, this proliferative aspect of *ET-1* is not only involved in developmental progress. For instance, increased *ET-1* expression has been demonstrated in a variety of solid tumors thereby promoting growth and inhibiting apoptosis in breast cancer while decreasing tumor cell differentiation (35-38). However, none of these reports described the causative effect of *ET-1* on hyperproliferative lesions.

In this study, *ET-1* transgenic mice displayed intense lobular proliferation in various grades in mammary glands during lactation. Further, alveolar organization was lost in parts of these areas (Figure 3). Suggesting the overexpression of *ET-1* might contribute to mammary tumorigenicity.

One indication of a neoplastic progression is the loss of normal tissue architecture, including polarity. Generally, primary breast carcinomas show a dramatic increase in the ratio of luminal to myoepithelial cells, and many invasive breast carcinomas essentially lack myoepithelial cells completely (39). After the *SMA* immunohistostaining, these histological observations pointed to lactational hyperplasia in *ET-1* transgenic mammary glands during the middle of the lactation period (Figure 3 G, H).

The lactational hyperplasia is known as a benign mammary lesion unique to pregnancy and lactation. This lesion may occur in response to the alteration of physiological conditions which represent pregnancy and lactation and it diminish spontaneously after the lactation (40,41).

A possible causative effect of *ET-1* overexpression on the development of lactational hyperplasia might have been mediated by the interaction of its *GPCR*s with the *EGFR*s, since *ET-1* is known to possess growth regulatory properties (3,8,42).

ET-1 exerts its effect by binding to two distinct *GPCR*, *ETAR* and *ETBR*. In addition to typical *GPCR* signal characteristics, these receptors are also able to communicate with structurally unrelated receptors such as the *EGFR*, the most prominent receptor tyrosine kinase (8,9), resulting in *EGFR* characteristic intracellular signals. The expression level of both endothelin receptors was determined using RT-PCR and Western blotting (Figure 2) and no difference could be detected between wild type and transgenic animals, suggesting that the increase in expression of *ET-1* is not influencing the expression of its receptors in this biological system.

Initial Western blot studies performed with mammary glands derived from *ET-1* transgenic mice were not able to detect an *EGFR* signal during the lactation period. Therefore, immunoprecipitation was employed to detect total *EGFR* and phosphor- *EGFR*. Structurally, *EGFR* contains multi phosphorylation sites in intracellular domains, and several tyrosine (Y) phosphorylation domains *(Y845, Y998 and Y1068)* were analyzed during initial studies on *EGFR* activation (data not shown). Figure 4 *ET-1* transgenic mice exhibits significantly more active *EGFR* phosphorylation on the specific domain tyrosine 845 residue. It has been reported that various *GPCR* agonists including *ET-1* could stimulate the phosphorylation of *Y845* in the *EGFR in vitro* (43).

Due to the technical difficulty to demonstrate the *EGFR* transactivation *in vivo*, we have analyzed also *ERK* protein activation which is the most well defined signalling pathway from the cell membrane to *ERK* 1 and *ERK* 2 is that mediated by the *EGFR* (44). *ET-1* stimulates *ERK* 1 and *ERK* 2 through cognate *GPCR* receptors by transactivation of the *EGFR* in various cell types and tissues (45,46). Additionally, increased *ERK* activation is documented in tubular hyperplasia as a result of *ET-1* induction (47,48). In Figure 4 C, D, E our Western blot studies represent that the transgenic group has significantly more active *ERK*1 and *ERK*2 during pregnancy and lactation stages which implies us *ET-1* induced *EGFR* transactivation *in vivo*.

To further characterize the assumed signaling network in *ET-1* transgenic mammary glands, the most widely expressed ligands *TGFa, EGF, HBEGF* and *amphiregulin*, involved in the activation of the *EGFR*, were analyzed using RT-PCR. As presented in the Figure 5 the *amphiregulin* expression was significantly upregulated in *ET-1* transgenic mice at lactation day 14 and pregnancy day 18 which is suggesting that the regulation of *amphiregulin* is influenced by the *ET-1* overexpression. None of the three other ligands did exhibit significant differences in their expression level.

It was demonstrated that *amphiregulin* is a unique *EGFR* ligand for ductal branching and ductal outgrowth (49). Normally, *amphiregulin* expression is increased during pregnancy and decreased dramatically after parturition (32,33). However, *amphiregulin* gene expression is upregulated in *ET-1* transgenic mice both during pregnancy and lactation periods.

Moreover, it has been noted that high *amphiregulin* expression is strongly associated with breast carcinomas and neoplastic progression (50,51). Several studies reported an increased *amphiregulin* expression in parallel to breast cancer. Therefore, *amphiregulin* was chosen as a pharmacological target for breast cancer treatment (52,53).

ADAM 17, the transmembrane metalloproteinase, is responsible for *amphiregulin* activation. This is the upstream of *EGFR* dependent of signal pathway (54). *ADAM* 17 was analyzed employing RT-PCR techniques. As shown in Figure 5C, *ADAM* 17 expression was significantly upregulated in *ET-1* transgenic mice during the lactation day 14.

The mechanism of *ET-1* induced *EGFR* transactivation might provide a molecular explanation how overexpression of *ET-1* could regulate the proliferative behaviour of tumor cells. Indeed, *ET-1* transgenic mice were found with an increased *EGFR* activation in their mammary glands during lactation. Moreover, *EGFR* activation is maintained by an increased *amphiregulin* expression (55) which again might be regulated by the observed *ET-1* overexpression.

Figure 6 represents mammary histology of both *ET-1* transgenic and wild type mice mammary glands at involution day 14. There was no residue of benign tumors found in *ET-1* transgenic mice. Probably apoptotic process was stronger than cell survival. Therefore, those benign lesions were diminished due to the absence of *ET-1* expression during the involution process. Since ET-1 expression does not take place within the involution we can not further elaborate tumorigenicity of ET-1in mammary gland physiology. However, the key components of signal transactivation (Figure 1) were found to be upregulated or more active while *ET-1* expressions were higher than normal state. This result suggests the causative effect of *ET-1* on pathophysiological alteration of the mammary glands taken place via *EGFR* transactivation.

Taken together, various studies underlining *amphiregulin* expressions and activation of *EFGR,* are positioned at the center of breast cancer or neoplastic transformation, as in this study, we focused on factors that might affect the regulation of this system. In this regard, the *ET-1* axis reaches an important point in the understanding of cancer biology by increasing and therefore activating the characteristic genes observed in neoplastic transformation. Indeed, recent studies confirm our thoughts on the development of anticancer drugs to block endothelial receptors (57,58).

In future studies, by further studying the properties of the signal downstream of the *ET-1* axis, we can find out more about the neoplastic transformation of the mammary glands on a larger scale.

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A Brief Reconnoitre about Effects of *MMP9* on Aortic Dissection

MMP9 Geninin Aort Diseksiyonundaki Olası Etkilerinin Araştırılması

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ABSTRACT

Objective: Matrix metalloproteinases (MMPs) are the extracellular matrix regulators that frequently investigate cardiovascular diseases and cancer metastasis. Our study aimed to examine specific polymorphisms in the *MMP9* gene in our patients with aortic dissection and compare the effect of *MMP9* on aortic dissection with expression datasets.

Materials and Methods: Q279R and P574R polymorphisms were analyzed in 44 aortic dissection patients and 40 healthy donors via polymerase chain reaction-restriction fragment length polymorphism. (PCR-RFLP) methods. Q279R and P574R prevalence was statistically compared with the medical data of the patients. Additionally, we collected datasets of aortic dissection from NCBI GEO to reanalyze GEO2R and RStudio to see metalloproteinase activity on samples. Later, enrichment analysis was processed on widely used databases.

Results: Genotypic distribution of alleles was similar in the two study groups. In addition to this, female CG carriers had a higher risk of developing aortic dissection than those of males. As the results of the protein-protein interaction analysis of *MMP9* and patients' clinical data, hypertension was found to be the significant outcome of P574R variation in the patients. In array analysis, *MMP9* expression did not change critically, but *TIMP*s had been downregulated in many samples. Also, *MMP9* targeted miRNA expression levels were detected as low in aortic tissue and blood.

Conclusion: Q279R and P574R are two polymorphisms that do not directly affect *MMP9* protein structure. Consequently, studied polymorphisms and performed meta-analysis show that *MMP9* does not spark off the phenotype but sets the stage for aortic dissection development as seen in the statistical results. Furthermore, enrichment analysis on datasets shows *MMP9* was not a primary reason for vascular remodeling.

Keywords: Aortic dissection, *MMP9*, Q279R, P574R, gene expression data

ÖZ

Amaç: Kardiyovasküler hastalıklar ve kanser metastazında sıklıkla araştırılan matriks metalloproteinazlar (MMPs) ekstraselüler matriks düzenleyicileridir. Çalışmamız, aort diseksiyonu olan hastalarda *MMP9* genindeki spesifik polimorfizmleri incelemeyi ve *MMP9*'un aort diseksiyonu üzerindeki etkisini ekspresyon veri setleri ile karşılaştırmayı amaçlamaktadır.

Gereç ve Yöntem: Q279R ve P574R polimorfizmleri 44 aort diseksiyon tanısı almış ve 40 sağlıklı bireyde polimeraz zincir reaksiyonu - restriksiyon parça uzunluğu polimorfizmi (PCR-RFLP) yöntemiyle çalışıldı. Q279R ve P574R prevalansı istatistiksel olarak hastaların tıbbi verileriyle karşılaştırıldı. Buna ek olarak, NCBI GEO veri tabanından aort diseksiyon veri setleri toplandı ve *MMP9* ifadesindeki farklılıkları görmek amacıyla bu veri setleri GEO2R ve RStudio ile yeniden analiz edildi. Elde edilen sonuçların informatik analizi için çevrimiçi veri tabanları kullanıldı.

Bulgular: CG alleli taşıyıcısı kadınların aort diseksiyonu geliştirme riski erkeklerden daha yüksek bulunmasına rağmen her iki çalışma grubunda da allellerin genotipik dağılımı benzer bulunmuştur. *MMP9*'un protein-protein etkileşim analizinin ve hastaların tıbbi verilerinin incelenmesinin sonucu olarak, P574R hipertansiyonu olan hastalarda önemli bir bulgu olarak değerlendirilmiştir. Array verisi analizinde ise *MMP9* ifadesinde kritik bir değişim gözlemlenmemiş olup, birçok örnekte *TIMP* ifade seviyelerinde azalma tespit edilmiştir. Ayrıca *MMP9*'u hedefleyen miRNA ekspresyon seviyelerinin aort dokusu ve kanda düşük olduğu saptanmıştır.

Sonuç: Q279R ve P574R, *MMP9* protein yapısını doğrudan etkilemeyen iki polimorfizmdir. İncelenen polimorfizmler ve gerçekleştirilen meta-analizler, *MMP9*'un fenotipi doğrudan etkilemediği, ancak istatistiksel sonuçlarda görüldüğü gibi aort diseksiyonu gelişimi için zemin hazırladığını göstermektedir.

Anahtar Kelimeler: Aort diseksiyon, *MMP9*, Q279R, P574R, gen ifade verisi

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INTRODUCTION

Aortic dissection is caused by the creation of a false lumen in the aorta. An intimomedial tear allows blood flow to enter the aortic wall by creating a false lumen or a secondary channel, called an aortic dissection, a catastrophic manifestation of the acute aortic syndrome. Aortic dissection has the highest mortality rate among cardiovascular diseases due to the rising of lethality rate at 1% per hour. Diagnosis of dissection is done by scanning the aorta and its branches with computed tomography (1-3). Predisposition to acute aortic dissection has been associated with atherosclerosis vasculitis, bicuspid aortic valves, male gender, long-term arterial hypertension, and collagen-based disorders such as Marfan's and Ehlers-Danlos syndromes (4-6).

Matrix metalloproteinases (MMPs) are extracellularly acting, zinc-dependent endopeptidases that break the peptide bonds of nonterminal amino acids. MMPs are involved in the developmental stages of the extracellular matrix and have been implicated in several collagen-based disorders (7,8). MMPs bind to adhesion and extracellular matrix proteins and have been divided into types based on their substrates, including membrane, gelatinase, stromelysin, matrilysin, and collagenase (9,10). *Matrix metalloproteinase 9* (*MMP9*), also known as the 92 kDa type IV collagenase or gelatinase B, is categorized in the gelatinase subgroup and has a significant role in extracellular matrix degradation (7,8). *MMP9* in humans contains an NH_2-terminal prodomain, a COOH-terminal hemopexin-like domain, a catalytic domain, and a linker domain. The *MMP9* gene is positioned at the chromosome region 20q11.2-13.1.8 and has 13 exons and 12 introns. *MMP9* is secreted from various types of cells, such as fibroblasts, macrophages, and neutrophils (8-11). *MMP9* is positively regulated by transcription factors (such as NF-kappaβ and activator protein-1) and polyomavirus enhancer A-binding protein-3. Tissue inhibitors of metalloproteinases inhibit *MMP9* by binding to the zymogen forms of the enzyme. Recent studies have revealed that *MMP9* polymorphisms are associated with cardiovascular diseases, such as hypertension, myocardial infarction, and atherosclerosis. The most thoroughly investigated polymorphisms in the literature are: C-1562T, Q279R, 836GA, R668Q, and P574R (8).

In this study, we investigated the MMP9, Q279R and P574R variations in Turkish aortic dissection patients. In addition, we analysed our results with the published epigenome, expression, and miRNA array data.

MATERIALS AND METHODS

Our study was designed as a retrospective case-control study, and a case group was built comprising 44 patients who underwent aortic dissection surgery between 2007 and 2011. The patient's diagnosis was confirmed with echocardiography and computed tomography imaging techniques that demonstrated an intimal flap with a true and a false lumen. As hypercholesterolemia, diabetes mellitus, hypertension, and hypertriglyceridemia are risk factors for aortic dissection, the patients' blood pressure, fasting glucose, triglyceride, and low-density lipoprotein levels were recorded. Iatrogenic, syndromic, and traumatic aortic dissection patients were excluded from the study. The control group consisted of 40 individuals with normal findings on their physical and echocardiographic examinations. This study has the ethics committee approval from Istanbul University (Istanbul Medical Faculty Clinical Research Ethics Committee; 2018/1252), and an informed consent was obtained from all selected individuals.

Genomic DNA was extracted from the peripheral blood of both the patient and control group subjects. The polymerase chain reaction (PCR) was used to amplify the targeted genomic sequences. The primers designed for the targeted regions are shown in Table 1. Amplification was conducted using a total volume of 25 µl comprising: 100 ng genomic DNA; a reaction buffer includes 25 mM magnesium chloride and 10X potassium chloride; 1 U Taq polymerase; 2.5 mM deoxynucleotide; and 10 pmol of each primer. The PCR thermal cycling steps were performed at 95°C for 2 minutes (initiation), 94°C for 30 seconds (denaturation), 55°C for 45 seconds (annealing), 72°C for 30 seconds (extension), and 72°C for 5 minutes (final extension). The PCR products were analyzed on a 2% agarose gel, and electrophoresis was performed at 80 V for 30 minutes. The prevalence of two single nucleotide polymorphisms of the *MMP9* gene, Q279R, and P574R, was evaluated in the patient and control groups. The Q279R polymorphism has three genotypes: the common genotype is AA, and the less common genotypes are AG and GG. The other studied single nucleotide polymorphism, P574R, has only two genotypes: the widespread genotype is CC, and the less common genotype is CG. The reaction products were digested with the MspI and BsrBI restriction enzymes. For the digestion step, 20 µl of PCR product was mixed with 5 µl of digestion mix including 1 µl dH_2O, 1.5 µl restriction enzyme, and 2.5 µl tango buffer. The samples were incubated at 37°C for 3 hours, and then electrophoresis was done for identification of the genotype.

Table 1. Details of enzyme digestion analysis

Polymorphisms	Primer	PCR Product (bp)	Restriction Enzyme
Q279R (rs17576)	5′-GGCCCAATTTTCTCATCTGAG-3′ 3′-GAGCTTGTCCCGGTCGTA-5′	292	MspI
P574R (rs2250889)	5′-CTTATCGCCGACAAGTGG-3′ 3′-GCACAAGACGTTTCGTGG-5′	190	BsrBI

The Statistical Package for the Social Sciences software for Windows (SPSS version 25, IBM Corporation, Armonk, New York, USA) was used to perform all the statistical analyses. The Hardy-Weinberg equilibrium-based genotypes distribution prediction was used to analyze the study and control groups. The genotype distribution frequencies of the patient and control groups were compared using Fisher's exact test. The clinical features of patients and their genotype characteristics were analyzed by one-way analysis of variance, Tukey's honest significant difference tests, and the independent sample t-test. The p values <0.05 were considered significant.

To perform the meta-analysis array data, datasets of aortic dissection patients had gotten NCBI GEO database. GSE84274, GSE52093, GSE98770 were used to analyze gene expression levels, GSE92427 was selected to explore miRNA levels on aortic dissection levels (12-15). Although datasets had more than one aortic tissue disease, we chose only the aortic dissection patients' data and control groups. First, calculations were performed through the medium of a ready-to-use GEO2R program. Then,we checked the results with the Bioconductor package of R. Top 100 genes, according to their adjusted *p*-value, were selected to analyze. Obtained gene functions and relation with *MMP9* were analyzed with STRING-db, UNIPROT, NCBI, PANTHER, and other commonly used databases (16-19).

RESULTS

The genotype distribution characteristics of the patient and control groups are shown in Table 2. Q279R and P574R were investigated in 84 and 77 individuals, respectively. Both the patient and control groups had similar percentages of males and females. There was a small genotype difference between the females and the males in the group of aortic dissection patients. More than half of the total number of individuals had widespread genotypes for two polymorphisms: the AA genotype was present in 56.8% of the patient group and 60% of the control group; the CC genotype was present in 87.8% of the patient group and 78% of the control group (AA for Q279R, CC for P574R) (p=0.943 for Q279R). Ancestral allele frequencies were different in the AD patients and dbSNP data for the two SNPs, however, they were not found statistically significant (p=0.721 for allele A, and p=0.384 for allele C). Statistical analysis revealed an association between the P574R and aortic dissection (odds ratio=0.486, p=0.361). Also, there was a different distribution of P574R between the male and female aortic dissection patients. Female individuals had a higher risk than males (odds ratio=0.750, p=0.361). However, in datasets, the male percentage is higher than females. (19 males vs 4 female patients)

The medications taken by the aortic dissection patients (n=44) with respect to the genotypes of Q279R and P574R are given in Table 3. Six different pharmaceuticals were selected, and their

Table 2. Genotypes of male and female subjects in the control and aortic dissection patient groups

		Q279R							P574R				
		AA		AG		GG			CC		CG		
		n	%	n	%	n	%	Total	n	%	n	%	Total
Control	Female	16	40	9	22.5	1	2.5	26	21	58.3	4	11.1	25
	Male	8	20	4	10	2	5	14	7	19.4	4	11.1	11
Patients	Female	19	43.2	12	27.3	3	6.8	34	27	65.8	4	9.7	31
	Male	6	13.6	3	6.8	1	2.3	10	9	21.9	1	2.4	10
p-value					0.943						0.361		

Table 3. Drugs currently used by aortic dissection patients with respect to the genotypes of Q279R and P574R

	Q279R					P574R			
	AA	AG	GG			CC	CG		
	n	n	n	Total	*p value*	n	n	Total	*p value*
β-blocker	13	7	4	24	0.487	20	3	23	0.732
ACE inhibitor	8	2	1	13	0.182	12	-	12	0.123
Calcium channel blocker	2	-	1	3	0.208	3	-	3	0.651
Acetylsalicylic acid	5	3	-	8	0.718	8	-	8	0.281
Angiotensin receptor blocker	3	3	-	6	0.426	6	-	6	0.402
Coumadin	5	2	-	7	0.621	5	1	6	0.598

(ACE: angiotensin-converting enzyme)

relationships with the polymorphisms were analyzed. It was clear that the angiotensin-converting enzyme (ACE) inhibitor, calcium channel blocker, and acetylsalicylic acid usage was in harmony with *MMP9* polymorphisms. (odds ratio=0.682 for coumadin, and odds ratio=0.952 for β-blocker).

According to their blood features, the genotype distributions of the aortic dissection patients are shown in Tables 4, 5, and 6.

The patients were grouped according to their genotypes. The mean and standard deviation values of the blood features and the widespread genotype for each group based on p-values and chi-squared analysis are given in Table 4. Blood glucose and potassium levels of the AG genotype as well as the alanine aminotransferase and platelet levels of the GG genotype were relative levels of significance.

Table 4. Laboratory findings according to Q279R genotypes in the patients group

	AA	AG	GG	*p-value*
Blood glucose	153.04±60.98	131.53±26.38	149.25±79.12	0.69
BUN	24.62±12.58	24.06±9.09	28.25±10.75	0.718
CR	1.51±1.66	1.35±0.57	1.45±0.46	0.967
AST	74.82±105.93	90.8±124.32	83.25±76.18	0.954
ALT	48.21±68.52	115.46±270.6	225.25±295.2	0.148
Sodium	140.78±3.9	140.66±3.71	143±5.35	0.461
Potassium	4.17±0.55	4.49±0.54	4.31±0.75	0.486
WBC	12.31±4.8	12.29±3.96	11.9±3.65	0.98
Hemoglobin	11.51±2.33	11.9±1.77	10.35±0.99	0.3
HCT	34.03±6.50	33.48±10	30.42±2.36	0.614
PLT	201.34±113.7	207.13±80.29	118.75±74.92	0.183
MPV	8.51±0.92	8.8±1.20	8.57±1.36	0.853
RDW	14.66±2.53	15.35±2.57	14.22±0.83	0.621

(BUN: blood urea nitrogen, CR: creatinine, AST: aspartate aminotransferase, ALT: Alanine aminotransferase, WBC: white blood cells, HCT: hematocrit, PLT: platelet, MPV: mean platelet volume, RDW: red cell distribution width. Data are given as mean ± standard deviation.)

Table 5. Statistical comparison (p-value) of laboratory findings according to Q279R genotypes in the patients group

	AA vs. AG	AA vs. GG	AG-GG vs. AA
Blood glucose	0.45	0.99	0.284
BUN	0.987	0.815	0.921
CR	0.93	0.996	0.734
AST	0.902	0.989	0.674
ALT	0.536	0.207	0.131
Sodium	0.996	0.562	0.762
Potassium	0.215	0.89	0.112
WBC	1	0.984	0.938
Hemoglobin	0.84	0.571	0.919
HCT	0.976	0.67	0.622
PLT	0.983	0.292	0.687
MPV	0.708	0.995	0.478
RDW	0.678	0.942	0.556

(BUN: blood urea nitrogen, CR: creatinine, AST: aspartate aminotransferase, ALT: Alanine aminotransferase, WBC: white blood cells, HCT: hematocrit, PLT: platelet, MPV: mean platelet volume, RDW: red cell distribution width.)

Alleles were compared with the blood features of the aortic dissection patients. The results of this comparison indicate that *MMP9* was associated with disease progenitors. There was a distinguishable difference between alleles A and G. The allele G carriers had a risk of developing aortic dissection compared to the allele A carriers.

According to the De Bakey classification, there are three different types of aortic dissection. Type 2 involves the ascending aorta, type 3 involves the descending aorta, and type 1 aortic dissection includes both. A large majority of our patients had type 1 aortic dissections. The genotype distribution of the studied single nucleotide polymorphisms across aortic dissection types is given in Table 7. Though there was no statistical significance between the two polymorphisms and aortic dissection type, P574R has a higher impact probability on aortic dissection than Q279R. After these statistical analyses, *MMP9* expression and methylation levels had not been changed on study groups of selected datasets in comparison with control groups as we expected—all these expression data were obtained from dissected aorta tissue that has the low secretion of gelatinase proteins.

Nevertheless, circulating miRNA levels had been determined by plasma, not from aortic tissue, and *MMP9* targeted miRNAs

Table 6. Laboratory findings according to P574R genotypes in the patients group

	CC	CG	*p-value*
Blood glucose	145.38 ± 57.62	137.4 ± 23.96	0.764
BUN	24.62 ± 11.2	19.2 ± 4.43	0.296
CR	1.47 ± 1.39	0.96 ± 0.18	0.416
AST	81.58 ± 111.7	35.8 ± 24.13	0.372
ALT	100.23 ± 211.9	31.6 ± 20.71	0.479
Sodium	140.64 ± 3.96	140.8 ± 3.56	0.936
Potassium	4.30 ± 0.56	4.25 ± 0.30	0.853
WBC	12.57 ± 4.55	9.7 ± 2.99	0.181
Hemoglobin	11.47 ± 2.19	12.52 ± 1.37	0.310
HCT	33.9 ± 6.25	31.3 ± 16.25	0.740
Platelet	201.7 ± 106.26	206.2 ± 68.74	0.928
MPV	8.68 ± 1.13	8.14 ± 0.43	0.301
RDW	14.72 ± 2.39	16.06 ± 2.93	0.264

(BUN: blood urea nitrogen, CR: creatinine, AST: aspartate aminotransferase, ALT: Alanine aminotransferase, WBC: white blood cells, HCT: hematocrit, PLT: platelet, MPV: mean platelet volume, RDW: red cell distribution width. Data are given as mean ± standard deviation.)

Table 7. Aortic dissection types and genotype distributions

	Q279R					P574R			
	AA	**AG**	**GG**	**Total**	***p value***	**CC**	**CG**	**Total**	***p value***
Type 1	22	13	4	39		32	4	36	
Type 2	1	-	-	1	**0.860**	1	-	1	**0.497**
Type 3	2	2	-	4		3	1	4	

were found downregulated. All miRNAs were checked from TargetScanHuman (version 7.2) and miRWalk (version 2.0) (20,21). 100 genes from each dataset were analyzed to find the biological pathways they relate to. *MMP9* expression did not critically change in studies, but regulators of *MMP9*, *TIMP1*, and *TIMP2* had been shown expression differences. However, upregulated genes were found to meet in a typical biological process, mitotic cell cycle regulation, and muscle cell construction. Downregulated genes play roles in cellular adhesion and developmental processes like angiogenesis. Also, these genes had a different affinity to chemicals and cofactors. Analysis results are given in Figure 1.

DISCUSSIONS

MMPs are known with their roles in collagen-based diseases, such as vascular rearrangement and devastation. They are divided into ligand subgroups, one of which is *MMP9*. A collagenase involved in extracellular matrix degradation, *MMP9* directly affects collagen-based structures and has been implicated in several vascular diseases. Research on the relationship between MMPs and aortic dissection has primarily focused on the concentrations of matrix metalloproteinase proteins and inhibitors (TIMPs) in plasma and tissue.

We investigated two amino acid changes, Q279R and P574R, located on different exons of the *MMP9* gene. The PolyPhen-2 prediction tool classifies Q279R and P574R as benign alleles (22) . Conformational changes in protein structure which result from Q279R and P574R variations are shown in Figure 2 (23-26). Moreover, *MMP9* and *TIMP1/2* interactions come into existence by hemopexin-like domains of *MMP9* and the C terminal of *TIMP*. P574R affects a hairpin structure in its place as a result of this docking of two proteins (27).

Insights into this structural information, we decided to focus on the proteins that bind directly to *MMP9*. In datasets GSE52093 and GSE84274, we ascertained proteins related to T cell regulation, hemopoiesis, angiogenesis, chemokine regulation, extracellular matrix binging, and vascular morphogenesis mission. Interestingly in the dataset GSE52093, some of the vital protein kinases which play roles in DNA damage, cell cycle, and apoptotic process are upregulated in dissected aorta tissue.

There was no significant association between genotypes and the patients' medications, which shows a tendency toward aortic dissection. Previous studies have shown that *MMP9* is strongly linked to hypertension due to its vascular remodel-

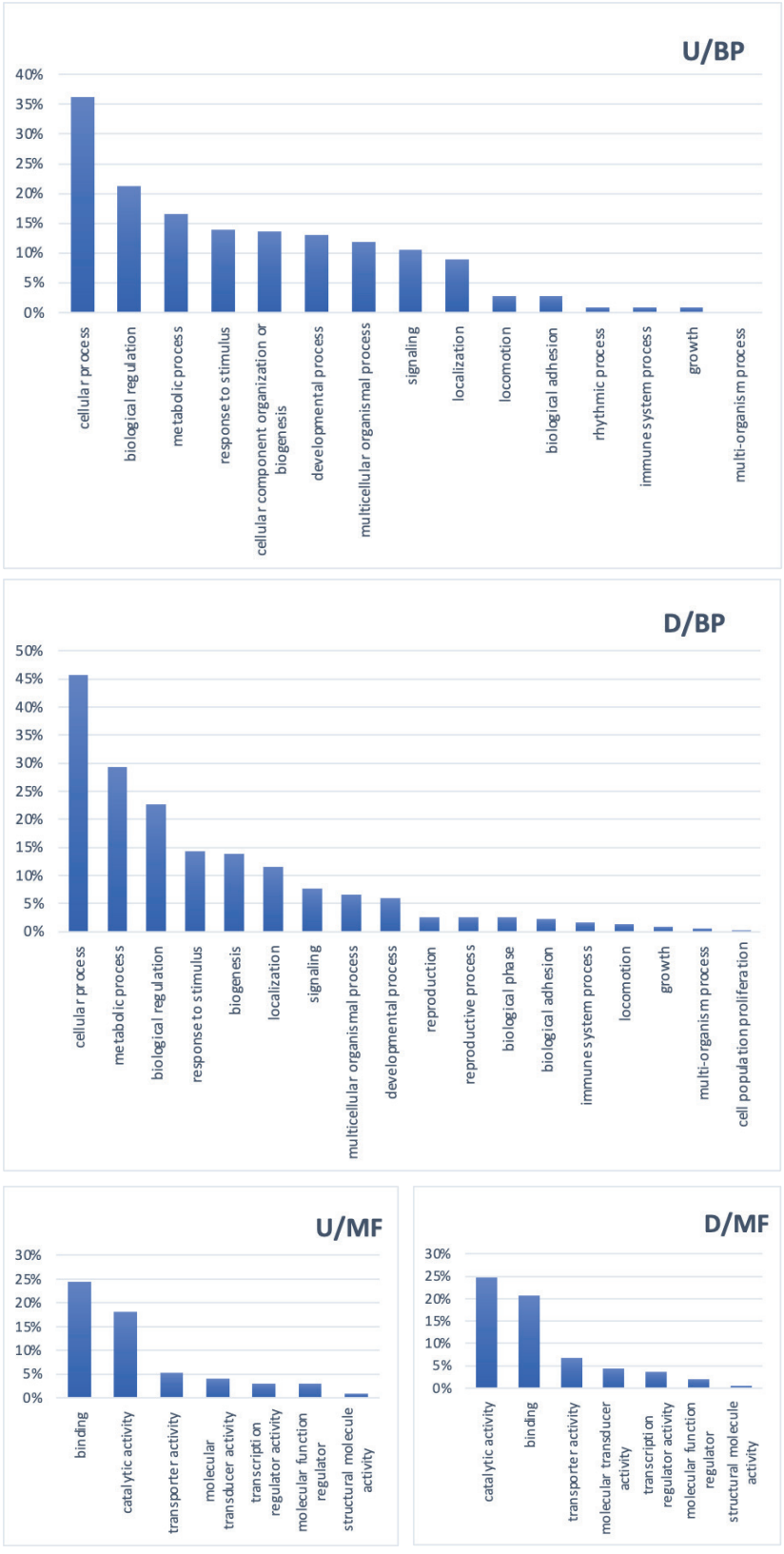

Figure 1. PANTHER enrichment results of all datasetsincluding top 100 upregulated and downregulated genes. (D: downregulated genes, U: upregulated genes, BP: biological process, MF: molecular function, P: pathway, PC: protein class).

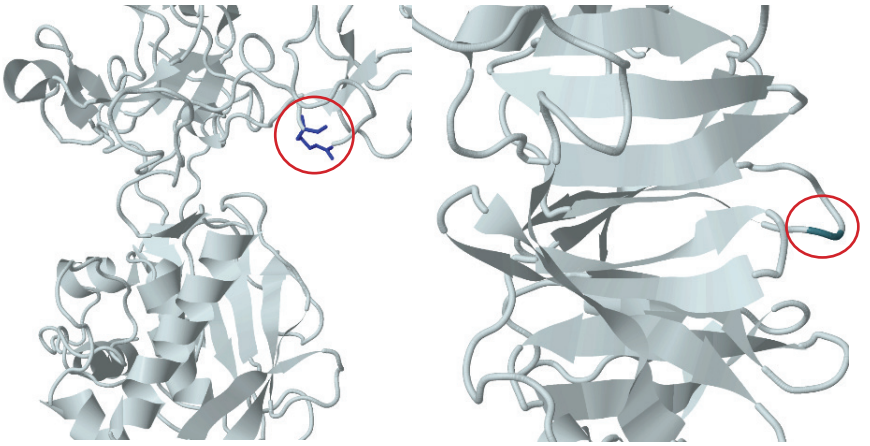

Figure 2. Secondary structures of mutant *MMP9* (1- 1L6J for Q279R, 2- 1ITV for P574R).

ing properties (28). In our study, one out of every three aortic dissection patients used drugs for hypertension, a significant risk factor for aortic dissection (29). Calcium channel blockers, ACE inhibitors acetylsalicylic acid usage differed significantly among the patients carrying the homozygous ancestral alleles. The patients carrying the G alleles showed obvious differences in alanine aminotransferase, potassium, platelet levels for those with Q279R, potassium, and white blood cell levels P574R. Our blood testing and genotyping analyses also demonstrated platelet differences. A correlation was found between the *MMP9* genotypes and acetylsalicylic acid, which blocks the blood's coagulation mechanism (30). Blood platelets and red blood cells play an essential role in coagulation. In the dataset GSE52093, JAK2 plays a role in blood coagulation expression level measured as downregulated. However, the function of *MMP9* in the coagulation mechanism is still unknown. Future studies can be designed to explore the effects of MMPs on the ion channels of the cell membrane.

MMP9 affects cell membrane structure and cell proliferation. The expression of the mitotic cell cycle regulator genes had different fold changes in aorta tissue. We may speculate that single nucleotide changes on *MMP9* might disrupt the cell membrane's calcium and potassium channels via binding regulatory proteins as *SULF2*. Calcium channels have different functions in different cell types; in cardiac tissue, calcium channels are involved in excitation-contraction coupling and pace-making properties (31). Potassium channels are directly associated with the calcium ions that activate the potassium channel. Both membrane channels are related to vascular conformation (32).

Recent studies have shown that MMPs break down nonmatrix proteins related to immune processes (33-35). The cells of the immune system not only protect the body against diseases and foreign invaders, but they also repair tissue injuries by proliferation and cell migration. These processes are triggered by chemotactic signals that are easily modified by MMPs. For instance, *MMP9* modulates T-cell function by cleaving the CD25 receptor of interleukin-2. *In vivo* experiments have shown that *MMP9* and a second MMP type, *MMP-2*, assist in T cell migration. *In vitro* experiments have indicated that high expression levels of *MMP9* and *MMP-2* lead to the increased migratory capacity of type 1 T helper cells. *MMP9* activity in both endothelial and T cells directly regulate the behavior of leukocytes and cytokine and chemokine formation. These environmental changes recruit white blood cells, an outcome supported by our study results showing that aortic dissection patients have high blood levels of leukocytes (34). In addition to these findings, aortic dissection types vary among patients. Also, *MMP9* targeted proteins like *MBP, RUNX2, ANGPT2*, and *SNAI2*, which have roles in blood cell migration and immune response regulation, had a different dissected aorta tissue expression.

In conclusion, *MMP9* is a collagenase that directly and/or indirectly influences vascular remodeling mechanisms. Patients carrying the G allele show an increased risk of aortic dissection than the ancestral allele carriers (A for Q279R and C for P574R) in accordance with RFLP results. Our study revealed the probable relationship between the cell membrane and *MMP9* proteins' ion channels with the Q279R and P574R polymorphisms. Our findings confirm that *MMP9* plays a role in aortic dissection so far as pharmaceuticals listed above explain how deformation occurs on the aorta. Aortic vessel deformations resulting from true or false lumens like aortic dissection lead to many cardiovascular diseases. Besides morphological deformation, chemicals also destroy vein structure. We can say that Q279R and P574R polymorphisms of the *MMP9* gene affect the extracellular matrix and cause vessel deformation in a roundabout way. Membrane organization defects might not result from *MMP9* polymorphisms, but these structural changes disrupt the molecular function of matrix metalloproteinase and form a basis of aortic dissection. These polymorphisms have been studied in the aortic dissection patient group for the first time in Turkey, and it will be a pioneering work for future studies.

Ethics Committee Approval: This study has the ethics committee approval from Istanbul University (Istanbul Medical Faculty Clinical Research Ethics Committee; 2018/1252), and an informed consent was obtained from all selected individuals.

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Effects of Exercise and Calorie Restriction on Brain and Testis in Natural Aging Model

Doğal Yaşlanma Modelinde Egzersiz ve Kalori Kısıtlamasının Beyin ve Testiste Etkileri

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ABSTRACT

The aim of our study was to examine the effects of exercise and calorie restriction on various tissue damage and antioxidant parameters in the brain and testis of rats in a natural aging model. For this purpose, male Sprague-Dawley rats were the control group (C), the elderly (A), the elderly with calorie restriction (ACR), the elderly who were exercised (AE) and the elderly who were exercised with calorie restriction (ACRE), they were divided into 5 groups. The control group was composed of three-month-old animals. The other group consisted of 15-month-old rats. Exercise and calorie restriction were applied for 6 weeks. At the end of the experiment, lipid peroxidation (LPO), nitric oxide (NO), glutathione (GSH) levels and superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and tissue factor (TF) were determined in brain and testicular tissues homogenates. As a result of the study, the A group's brain and testis LPO, NO levels and TF activity increased, GSH levels and SOD, CAT and GST activities decreased, when compared to the C group. As a result of our study, an increase in oxidant damage was observed with TF activity in the brain and testis in the natural aging model, and positive effects of exercise and calorie restriction on the antioxidant levels in the brain were determined, especially in aging.

Keywords: Aging, brain, testis, oxidant, antioxidant

ÖZ

Amacımız doğal yaşlanma modelinde egzersiz ve kalori kısıtlamasının sıçanlarda beyin ve testiste çeşitli doku hasarı ve antioksidan parametreler üzerine etkilerinin incelenmesidir. Bu amaçla Sprague-Dawley sıçanlar kontrol (K), yaşlı (Y), kalori kısıtlaması uygulanan yaşlı (YKK), egzersiz uygulanan yaşlı (YE) ve kalori kısıtlaması ile egzersiz uygulanan yaşlı (YKKE) olmak üzere 5 gruba ayrıldı. Kontrol grubu üç aylık genç hayvanlardan oluşturuldu. Diğer gruplar 15 aylık sıçanlardan oluşturuldu. Egzersiz ve kalori kısıtlaması 6 hafta boyunca uygulandı. Deney sonunda beyin ve testis dokuları homojenatlarında lipit peroksidasyon (LPO), nitrik oksit (NO), glutatyon (GSH) düzeyleri ile süperoksit dismutaz (SOD), katalaz (CAT), glutatyon-S-transferaz (GST) ve doku faktörü (DF) aktiviteleri tayin edildi. Çalışma sonucunda K grubu ile kıyaslandığında Y grubunda, beyin ve testis LPO, NO düzeyleri ve DF aktivitelerinin arttığı, GSH düzeyleri ile SOD, CAT ve GST aktivitelerinin ise azaldığı gözlendi. Doğal yaşlanma modelinde beyin ve testiste DF aktivitesi ile beraber oksidan hasarda artış gözlenmiş olup, özellikle yaşlanmada egzersiz ve kalori kısıtlamasının beyinde antioksidan düzeyleri üzerinde olumlu etkileri belirlenmiştir.

Anahtar Kelimeler: Yaşlanma, beyin, testis, oksidan, antioksidan

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INTRODUCTION

The aging process is physiologically observed in every living organism, and in this process, an irreversible decline occurs in all functions at the level of tissue, cell and molecule. Among the general signs of aging, a decline can be seen in body functions, adaptation to environmental changes and resistance to harmful factors. In addition, due to aging, changes in cell structure and a decrease in the number of cells and the regeneration ability of the body are observed (1).

Aging is a process that is too complex to be defined by a single mechanism. The biological mechanisms involved alone are not explanatory and are often at the theoretical level. However, not all organs in the organism age at the same rate and no organ ages in the same way in different individuals of the same species. This situation can be explained by individual variations in aging. Today, there are different theories and hypotheses about aging. Increasing oxidative damage has been closely associated with aging and the development of aging-related diseases. Therefore, oxidative stress has an important place in aging and related diseases. Free radicals are molecules produced by aerobic metabolism in a continuous manner and with high proportion. These radicals cause damage in macromolecules such as DNA, proteins and lipids (1,2).

Exercise is defined as all physical activities performed in order to improve and maintain physical and mental health and to gain physical fitness. Exercise, when done regularly, has very important benefits on human health. Exercise improves the functions of circulatory, respiratory, digestive, excretory and skeletal-muscular systems. Inactivity causes various health problems (3). Regular exercise has been associated with a reduction in overall mortality and morbidity in the middle-aged and elderly (4).

Calorie restriction is the limitation of food intake so as not to cause nutritional deficiency, slowing down the aging process. The pathologies associated with aging have also become an important issue in gerontology in recent years. After revealing the relationship between calorie restriction and increase in life span, studies have been conducted on the mechanisms by which calorie restriction regulates aging. The belief that calorie restriction has an "anti-aging" effect, or that it is an antidote to the aging process, has gained wide popularity. However, the relationship between classic calorie restriction and the aging process has not yet been clarified. It is not yet known exactly how to establish optimal food intake for different genotypes (5).

Although aging is an inevitable process, it can be affected by many factors. The ways in which different organs age are also different from each other. Therefore, pathological conditions that cause structural damage and cognitive impairment in the central nervous system during the aging process are also closely related to aging (6). However, the amount of calories taken with nutrition and the level of exercise can be associated with the brain aging through general metabolic activities (7). It has been shown in several studies that oxidative stress can reduce male infertility (8-10). The aim of our study was to examine the effects of exercise and calorie restriction on various tissue damage and antioxidant parameters in the brain and testis in rats in a natural aging model.

MATERIAL AND METHODS

Sprague Dawley type rats (300-350 g) obtained from Marmara University DEHAMER Animal Laboratory were used in our study. The local ethics committee approved the experimental procedures (130.2013.Mar). The experimental animals were divided into 5 groups. There were 6 rats in the control (C) and aged (A) groups, and 10 rats in the elderly and calorie restriction (ACR) groups, aged and exercise group (AE) and aged group with calorie restriction and exercise (ACRE). The C group consisted of 3-month old rats that were fed a standard diet. The A group consisted of 15-month-old rats that were fed a standard diet. The ACR group consisted of 15-month-old rats that were given a calorie restricted for 6 weeks. The AE group consisted of 15-month-old rats that were given swimming exercise for 6 weeks. The ACRE group consisted of 15-month-old rats that were exercised for 6 weeks with calorie restriction. Calorie restriction was applied by reduction of feeding by 40% from the standard feed. For exercise, rats were floated for 30 minutes, 3 days a week for 6 weeks in a warm pool of water (25±2^{0}C), previously filled with warm tap water. At the end of the 6 weeks experimental period, decapitation was performed under ether anesthesia and the brain and testis tissues were removed and washed with saline. They were cleaned of membranes and veins and divided into small pieces in ice. These small pieces were then cut into minced meat with surgical scissors and weighed. Tissues were homogenized by adding saline (mL) equal to their weight, and tissue homogenates of 10% were prepared.

To measure the levels of proteins, copper ions were applied to the rats in an alkaline environment. They were then reduced with a phosphomolybdic-phosphotungstic acid reagent (folin reagent). The intensity of the blue color was evaluated spectrophotometrically. The intensity of the blue color formed is proportional to the protein concentration (11). The absorbance of the pink color that was produced at the end of the reaction of the lipid peroxidation (LPO) product malondialdehyde (MDA) and thiobarbituric acid (TBA) was evaluated spectrophotometrically to determine LPO (12). Tissue factor (TF) activity was determined by utilization of the quick method using plasma taken from healthy people. Tissue homogenate is used as a source of tissue factor (Thromboplastin). The time elapsed for fibrin formation after the addition of calcium chloride was determined. Activity varies inversely with time (13). Tissue factor activity is inversely proportional to the time taken for clot formation. To measure nitric oxide (NO) levels, nitrate was converted to nitrite with vanadium (III) chloride. The complex diazonium compound was produced based on the reaction of nitrite sulfanilamide with N- (1-Naphthyl) ethylenediamine dihydrochloride in an acidic medium. This colored complex formed was measured spectrophotometrically at 540 nm (14). The colored production of the reaction of Ellman separator, 5-5 'dithiobis 1-2 nitro benzoic acid (DTNB) and sulfhydryl groups was spectrophotometri-

cally determined for reduced glutathione (GSH) determination (15). superoxide dismutase (SOD) activity was measured as the ability of riboflavin sensitized o-dianisidine to increase the rate of photooxidation (16). Catalase (CAT), activity was determined based on the formation reaction of H_2O_2 to H_2O (17). Glutathione-S-transferase (GST) activity was measured according to the spectrophotometric determination of the 340 nm absorbance belonging to the product produced by conjugation of GSH with 1-chloro-2,4-dinitro-benzene (CDNB) (18). Analysis of variance (ANOVA) followed by Tukey's multiple comparison test were used for statistical analysis (GraphPad Prism 9.0, California, USA).

RESULTS

Brain Tissue

GSH values in brain tissue were decreased in A and ACR groups when compared with the controls. Compared to the A group, it was increased in the ACR, AE and ACRE groups. GSH values were increased in the ACRE group compared with the ACR group. SOD activity was significantly decreased in the A, AE, ACR and ACRE groups compared to the controls. In comparison with the A group, it was increased significantly in the AE, ACR and ACRE groups. CAT activity was decreased in the A, AE and ACR groups when compared to the C group, and it was increased significantly in the ACRE group compared with the A group. GST activity was decreased in the A group in comparison with the C group, and significantly increased in the ACRE group compared with A and AE groups (Figure 1). LPO was increased significantly in A and AE groups compared to the C group. LPO was decreased significantly in the ACR group compared with the A group. Brain TF activity was increased significantly in A, AE and ACRE groups compared with the C group. NO values were increased significantly in the A, AE and ACRE groups compared with the C group. Compared to the A group, NO values was decreased in the ACR group. NO values were increased in the ACRE group compared with the ACR and A groups (Figure 2).

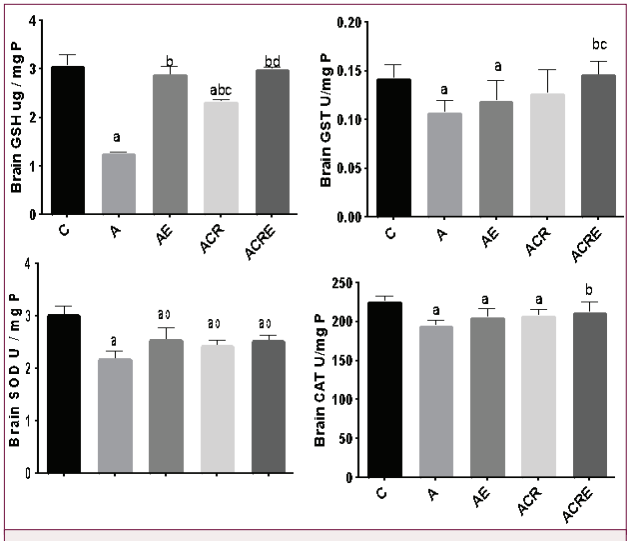

Figure 1. Brain GSH levels, GST, SOD and CAT activities of the groups. [a]p<0.05 significantly different than the C group; [b]p<0.05 significantly different than the A group; [c]p<0.05 significantly different than the AE group; [d]p<0.05 significantly different than the ACR group.

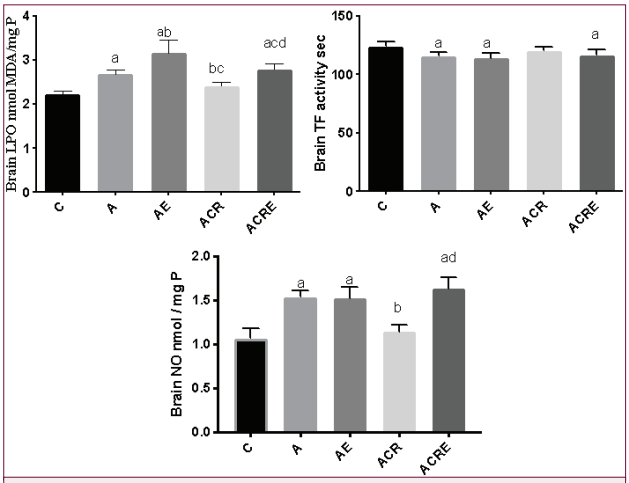

Figure 2. Brain LPO, NO levels, and TF activities of the groups. [a]p<0.05 significantly different than the C group; [b]p<0.05 significantly different than the A group; [c]p<0.05 significantly different than the AE group; [d]p<0.05 significantly different than the ACR group.

Testis Tissue

Testis GSH levels were decreased significantly in the A, AE, ACR and ACRE groups, compared to the C group. There was a significant increase in the GSH levels of ACRE group compared with

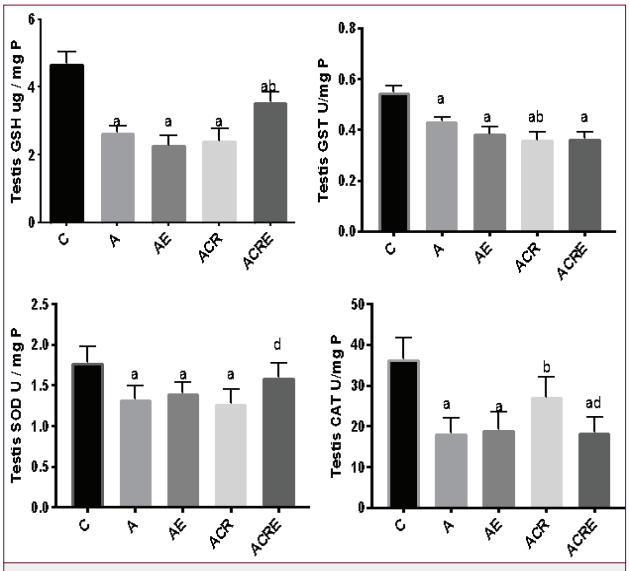

Figure 3. Testis glutathione levels, GST, SOD and CAT activities of the groups. [a]p<0.05 significantly different than the C group; [b]p<0.05 significantly different than the A group; [c]p<0.05 significantly different than the AE group; [d]p<0.05 significantly different than the ACR group.

the C and A groups. SOD activity was decreased significantly in the A, AE, ACR groups compared with the C group, and was also increased significantly in the ACRE group compared with the ACR group. CAT activity was decreased significantly in the A, AE, and ACRE groups compared to the C group, and increased significantly in the ACR group compared with the A group. GST activities were decreased significantly in the A, AE, ACR and ACRE groups compared with the C group (Figure 3). LPO was increased significantly in the A group compared with the C group, and was decreased significantly in the AE, ACR and ACRE groups compared with the A group. In the ACRE group LPO was also decreased in comparison with the A, AE and ACRE groups. TF was increased significantly in the A, AE, ACR, and ACRE groups compared with the C group. NO levels were increased significantly in the A, AE and ACR groups compared with the C group, and were decreased significantly in the AE and ACRE groups compared with the A group (Figure 4).

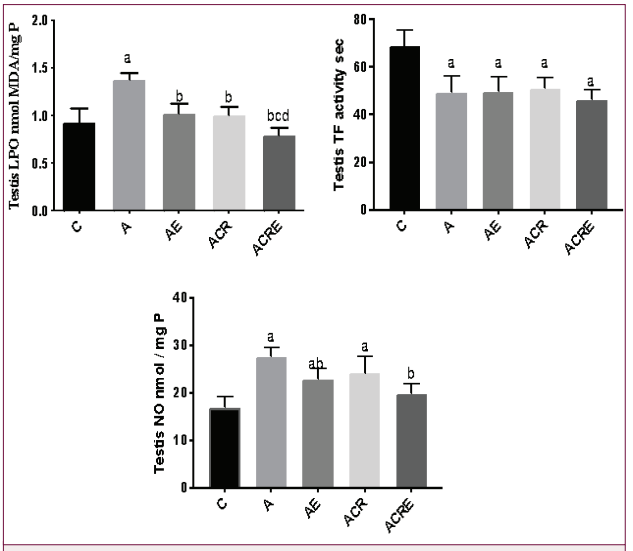

Figure 4. Testis LPO, NO levels, and TF activities of the groups. [a]p<0.05 significantly different than the C group; [b]p<0.05 significantly different than the A group; [c]p<0.05 significantly different than the AE group; [d]p<0.05 significantly different than the ACR group.

DISCUSSION

As a result of our study, LPO, NO levels and TF activity were found to have increased in the brain and testicular tissues of the elderly group compared with the control group. The GSH levels and SOD, CAT and GST activities were found to have decreased. Compared with the elderly group, LPO, NO levels and TF activity were decreased, and GSH and SOD activity were increased in the brains of the elderly group with calorie restriction. LPO and GSH levels and SOD activity were increased in the elderly group that was exercised. SOD, CAT and GST activities were increased in the elderly group that were exercised together with calorie restriction compared to the elderly group. Compared to the elderly group, LPO and NO decreased in the testicles of the elderly group with exercise and calorie restriction, while calorie restriction increased CAT activity and decreased GST activity in the elderly group.

Although the basic principles and mechanisms of aging have not been fully elucidated, there are various theories on this issue. Increasing oxidative damage has been closely associated with aging and the development of aging-related diseases (19). Therefore, oxidative stress has an important place in aging and related diseases. Free radicals are molecules produced by aerobic metabolism in a continuous manner, with high proportion. These radicals cause damage to macromolecules such as DNA, proteins and lipids (20).

As the redox balance deteriorates with aging, more free radicals are formed. Oxidative protein damage due to increased oxidative stress may cause changes in the structure of proteins such as aggregation, fragmentation, and secondary / tertiary structure. All these changes may cause both susceptibilities to proteolysis and changes in the normal function of proteins (19).

With the emergence of the relationship between free radicals and aging, the search for a diet that reduces or stops oxidative stress has come to the fore. It has been shown that antioxidant-rich diets result in less weight gain and tumor formation in experimental animals and reduce autoimmune diseases (21).

Older animals were shown to have more mitochondrial reactive oxygen species (ROS) than young animals. Oxidative DNA damage is cited as one of the most significant causes of aging. ROS could cause damage to the sugar-phosphate skeleton in DNA and can also cause point mutations on bases (22).

With aging, oxidative mitochondrial DNA (mtDNA) damage occurs in striated muscles, heart muscles and the brain. The importance of oxidative mtDNA damage and related mutations in the aging process has been emphasized in various studies, and it has been shown that mitochondria plays a major role in aging. There are antioxidant defense systems that protect against oxidative damage in the organism. Whether the antioxidant defense systems in the tissues are more active depending on the ROS produced continuously in the long-lasting species or not is a separate research subject. A study found a relationship between SOD activity in the liver, brain and heart tissues and the maximum lifespan in 14 mammal species (23).

The effects of aging on the testicles in rats were studied . Testicular sections taken from 3, 6 and 24 month old rats were examined morphologically, and it was reported that Leydig cells and collagen fibers in the testicular interstitium were more common in 6-24 month old rats than in 3 month old rats . It has also been shown that macrophages are greater in 6 month old rats than in 3 and 24 month old rats. Testosterone and luteinizing hormone (LH) levels did not differ between 3 and 6 month old rats, but it was reported to be lower at 24 months (24).

In a study examining the antioxidant activities of rat testis, it was suggested that the testicular antioxidant system is differ-

ent from the liver. The most significant difference was reported as high SOD activity in the testis of 6-10 day old rats, and low CAT and GSH-Px activity in the testis. It has been suggested that this high SOD ratio will make the testicles more susceptible to prooxidant factors. However, non-enzymatic antioxidants are also important in the defense system, but were not investigated in this study. The greatest difference in antioxidant enzyme activities in testicles was determined between 20-30 day old rats and it has been reported that this difference may be due to the change in testicular structure. Fetal type Leydig cells are replaced by adult Leydig cells shortly after birth. Antioxidant enzyme activities have been reported in different testicular cells. For example, SOD and GST activity is high in Sertoli cells. The reason for the high SOD activity in young rats has been defined as the increased need for hydrogen peroxide, which has not yet been fully elucidated in some developmental processes. Hydrogen peroxide has been shown to stimulate prostaglandin synthesis in the renal glomerulus in rats. Hydrogen peroxide has also been shown to stimulate cell differentiation. The high SOD activity seen in the early stages of life has been associated with the role of hydrogen peroxide in cell differentiation (25).

Only two known forms of SOD, cytosolic (Cu/Zn) and mitochondrial (Fe/Mn), are not found in the testicles. Testis also contain extracellular SOD that is generated by the sertoli and germ cells. It is well known that cytokines such as interleukin 1α stimulate extracellular superoxide dismutase (SOD-Ex) release in Sertoli cells (26).

It has been reported that catalase does not have much effect on the testis, and GPx, which uses GSH as an electron source to reduce H_2O_2 to water, has different isoforms in this tissue. GPx has been detected in mitochondria, nucleus and spermatozoa. Since many forms of GPx are selenium dependent, selenium deficiency affects male reproductive health negatively (27). It has been reported that physical exercise increases testis antioxidant enzyme activities in aged rats and may delay the effects that may occur on the testis with aging (28). In addition, it has been suggested that moderate exercise has a healing effect against oxidative damage in the testis due to chronic ethanol consumption (28).

However, it has been shown that excessive exercise increases lipid peroxidation in the testicles and can decrease antioxidant enzyme activities including CAT, SOD, and GST (29). The resulting stress can inhibit both steroidogenesis and germ cell differentiation in testis. The improvement of these effects when alpha tocopherol was administered as an antioxidant showed that exercise-induced testis dysfunction was associated with oxidative stress (30).

Aging is a biological process seen in all living organisms. Anatomical and physiological processes that occur with aging affect the development and outcome of some diseases. With the increase of the elderly population in the world, it is increasingly necessary to develop treatment programs for a better quality of life and prevention of diseases in this process. In the aging brain and various neurodegenerative diseases, there is a decline in normal antioxidant defense mechanisms, which increases the vulnerability of the brain to the harmful effects of oxidative damage. For example, the antioxidant enzymes SOD, CAT, GPx and glutathione reductase show reduced activities in the brains of patients with Alzheimer's disease (19).

Calorie restriction has been evaluated as the only reproducible experimental manipulation to increase life expectancy in many species. Restriction of nutrient intake below 30% to 50% of *ad libitum* levels during the early growth phase of life has been shown to result in significant increases in life expectancy of a variety of species, including insects, mice, fish, and rats (30).

In the literature, increased oxidative stress and inflammation with aging have been associated with an increase in TF expression (32,33). As a result of our study, increased TF activity in the brain and testis in the natural aging model was related to increased oxidant damage and inflammation with age. Especially in aging, calorie restriction is associated with TF and t he mechanism of its positive effects on NO needs to be elucidated.

Oxidative stress is closely related to the pathology of many diseases, especially aging, cancer, heart diseases and diabetes. For a quality and long life, all the functioning and repair mechanisms of the organism must work completely. Although acute physical activity has been shown to increase oxidative stress, regular physical activity has shown positive effects on aging-related diseases by strengthening antioxidant defense systems. In our study, the effects of calorie restriction, especially in reducing oxidative stress in the brain and strengthening antioxidant defense systems, were observed. Although calorie restriction can be considered as a way to increase functional lifespan, nowadays, there is evidence that choosing whole foods in a balanced way or addition of antioxidants to the diet is beneficial for a longer functional life span.

Ethics Committee Approval: All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Marmara University (130.2013.Mar).

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Protective Effect of Chard Extract on Glycoprotein Compounds and Advanced Oxidation Protein Product Levels in Diabetic Rat Livers

Diyabetik Sıçan Karaciğerinde Pazı Ekstresinin Glikoprotein Bileşikleri ve İleri Oksidasyon Protein Ürün Seviyeleri Üzerindeki Koruyucu Etkisi

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ABSTRACT

Objective: Diabetes mellitus is a metabolic disease of global importance. It affects many people by reducing their quality of life and even causing death. Therefore, research on diabetes mellitus maintains its popularity and continues to develop. Chard (*Beta vulgaris* L. var. cicla) is commonly used in diets and is known to have alternative hypoglycemic effects in diabetic individuals. This study investigated the protective effects of chard on glycoproteins (hexose, hexosamine, fucose and sialic acid) and the advanced oxidation protein product levels in diabetic rats' livers.

Materials and Methods: For this experiment, male Sprague–Dawley rats were separated into three groups: the control; Streptozotocin (STZ)-induced diabetic rats; and STZ-induced diabetic + chard extract. Fourteen days after diabetes induction, chard extract (2 g/kg/day, gavage) was administered for 45 days. On day 60, liver samples were collected, and 10% (w/v) homogenate was prepared for the analysis of glycoprotein components and advanced oxidation protein products.

Results: Levels of glycoprotein parameters that include hexose, hexosamine, fucose, and sialic acid, as well as advanced oxidation protein product levels, increased in the diabetic group. Chard extract administration curatively reverted the altered biochemical parameters.

Conclusion: From the results obtained, it can be suggested that chard extract has a protective effect on the diabetic livers of rats.

Keywords: Diabetes, chard, liver, glycoprotein

ÖZ

Amaç: Diyabet, yaşam kalitesini düşüren ve hatta ölüme neden olan, tüm dünyada birçok insanı etkileyen metabolik bir hastalıktır. Bu yüzden diyabet üzerine yapılan araştırmalar popülerliğini korumakta ve gelişimini devam ettirmektedir. Pazı (*Beta vulgaris* L. var. cicla) Türkiye'de şeker hastalarının alternatif hipoglisemik ajan olarak kullandığı bir bitkidir. Bu çalışmada pazı ekstraktının, diyabetik sıçanların karaciğer dokusunda bulunan glikoproteinler (heksoz, heksozamin, fukoz ve siyalik asit) ve ileri oksidasyon protein ürün seviyeleri üzerindeki koruyucu etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Deneyde kullanılan erkek Sprague-Dawley sıçanları 3 gruba ayrıldı. Kontrol sıçanları; Streptozotosin (STZ) ile diyabetik yapılan sıçanlar; pazı ekstresi verilen STZ diyabetik sıçanlar; Sıçanların diyabet olmasından on dört gün sonra, sıçanlara pazı ekstresi (2 g/kg/gün, gavaj ile) belirtilen dozda 45 gün uygulandı. 60. günde sıçan karaciğerleri çıkarıldı ve glikoproteinler ve ileri oksidasyon protein ürünlerinin analizi için %10 (w/v) karaciğer homojenizatları hazırlandı.

Bulgular: Yapılan deneyler sonucunda diyabetik sıçanların glikoprotein parametreleri olan heksoz, heksozamin, fukoz, siyalik asit ve ileri oksidasyon protein ürün seviyelerinin arttığı bulunmuştur. Pazı ekstresinin uygulaması, incelenen biyokimyasal parametre değerlerinin tersine çevirdiği belirlenmiştir.

Sonuç: Elde edilen sonuçlardan pazı ektresinin diyabetik sıçanlar üzerinde koruyucu bir etkiye sahip olduğu ileri sürülebilir.

Anahtar Kelimeler: Şeker hastalığı, pazı, karaciğer, glikoprotein

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INTRODUCTION

Diabetes mellitus is a serious disease of worldwide importance. It is caused by genetic factors, carbohydrate-heavy diets and lifestyles, and/or obesity. In the USA, it is projected that diabetes mellitus will be increased by 54% between 2015 and 2030, and its resultant death rate will increase by 38% annually (1). These statistics show that diabetes mellitus will increase year by year. For that reason, diabetes and the need for new treatment methods maintain their popularity in terms of research.

In diabetes mellitus, patients are not able to synthesis or use insulin sufficiently; therefore, blood glucose levels remain high (2). Glycoproteins are carbohydrate-linked proteins found on the cell surface. These compounds have important biological roles and are ubiquitous components of hormones, enzymes, membranes, and antibodies. The increasing interest in the biological importance of glycoproteins is indirectly or directly related to some diseases, including diabetes mellitus (3,4). There are fucose, sialic acid, hexose, and hexosamine in glycoproteins and glycosaminoglycans. They serve as biomarkers for some biological events such as secretion and absorption of macromolecules and cell differentiation, as well as recognition and membrane transport (5). Evidence has shown that people with diabetes have altered concentrations of glycoproteins when compared to non-diabetics. Some studies suggest that tissues like those in the liver and kidneys have elevated levels of glycoproteins due to insulin deficiency and high levels of blood glucose (6).

Diabetes mellitus can give rise to oxidative stress and carbonyl stress, thus leading to protein oxidations. Protein oxidation occurs under the influence of chlorinated oxidants, such as hypochlorous acid and chloramines. Among the important markers of protein oxidation, advanced oxidation protein products (AOPP) occur. Accumulation of AOPP in tissues has an important role in long-term diabetes; it is an important marker for determining diabetic damage (7).

Medicinal plants are an important aspect of alternative medicine; their continued popularity and use are linked to their valuable therapeutic functions and compositions/agents. They are valuable alternatives in the treatment of diabetes (8). Chard (*Beta vulgaris* L. var cicla, chenopodiaceae family) is one of the vegetables grown widely in Turkey, North India, South America, Mediterranean countries, and the USA. Some studies have shown that chard extracts exhibit diverse biological effects, including anticancer and anti-inflammatory (9), antimicrobial (10), hepatoprotective (11), antioxidant and anti-acetylcholinesterase (12), and anti-diabetic (13,14) activities. These biological activities are closely associated with the phytochemical composition/content of chard. *B. vulgaris* is reported to contain some saponins, flavonoid glycosides (15), flavonoids, vitamin C, vitamin E, carotenoids and minerals (16), folic acid, phospholipid, glycolipid, and some fatty acids (11).

This study investigated the effects of chard on glycoproteins and the advanced oxidation protein product levels in diabetic rats' livers.

MATERIALS AND METHODS

Preparation of Chard Extract

Chard leaves were purchased from a local market in Istanbul, Turkey. The chard plant was inspected by Prof. Dr. Neriman Ozhatay (Faculty of Pharmacy, Istanbul University). For the extraction of chard leaves, 100 g of dry chard leaves were weighed and boiled in 1 liter of distilled water for 8 hours. The water in the extract was then removed using an evaporator under reduced pressure.

Experimental Animal Design

The male Sprague-Dawley rats of 6-7 months old, weighing 380-420 g, were chosen to be used in this experiment. Ethical approval was obtained from Marmara University Animal Care and Use Committee (No: 68.2008.mar). The animals were grouped as follows:

Control rats (C) given citrate buffer (n= 8).

STZ-induced diabetic rats (D), (n= 8).

STZ-induced diabetic rats given chard extract (D+Chard), (n= 8).

The rats were made diabetic by intraperitoneal administration of 60 mg/kg STZ dissolved in 0.01 M citrate buffer (pH=4.5). A dose of chard extract (2 g/kg/day, gavage) was administered to the rats for 45 days, 14 days after diabetes. Liver samples were taken on the 60th day.

Biochemical Assays

The blood glucose levels of the rats were determined by an automatic glucose analyser 18 hours after STZ administration. The data/results have been previously published (14). Rats with fasting blood glucose greater than 200 mg/dl were considered to be diabetic. The liver tissues taken were homogenized (10% (w/v)) in ice-cold saline. From the liver tissue homogenates, hexose, hexosamine, and fucose levels were quantified by the methods of Winzler (17) and Dische and Shettles (18). For the sialic acid levels, the method of Lorentz et al. was used (19). Liver AOPP contents were assayed by the method of Witko-Sarsat et al. (20). The total protein level was determined according to the Lowry method (21).

Statistical Analysis

The GraphPad Prism 6.0 program (GraphPad Software, San Diego, California, USA) was used for statistical analysis. All data were expressed as means±standard deviation (SD). The results were calculated using t-test and analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *P*-values below 0.05 were considered statistically significant.

RESULTS

In experimentally induced diabetes, it was statistically evaluated whether the damage caused by diabetes was lessened or not by the application of chard. The significance between groups was compared using the one-way ANOVA test, and it was decided that it was significant when the p-value was equal

to or below 0.05. The lower the values specified, the higher the significance of the changes in the tissue would be.

Liver tissue glycoprotein levels are presented in figures 1-4. The levels of hexose ($p \leq 0.0001$), hexosamine ($p \leq 0.01$), fucose ($p \leq 0.05$), and sialic acid ($p \leq 0.001$) were increased significantly in the diabetic groups. The administration of chard extract to the diabetic group reversed these changes in the liver ($p \leq 0.0001$, $p \leq 0.001$, $p \leq 0.001$ and $p \leq 0.01$, respectively).

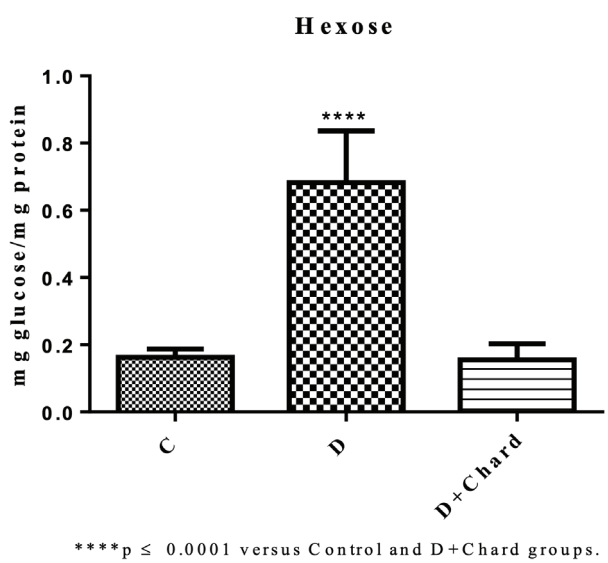

Figure 1. Liver tissue hexose levels of all groups. C (Control rats); D (STZ-induced diabetic rats); D+Chard (STZ-induced diabetic rats given chard extract).

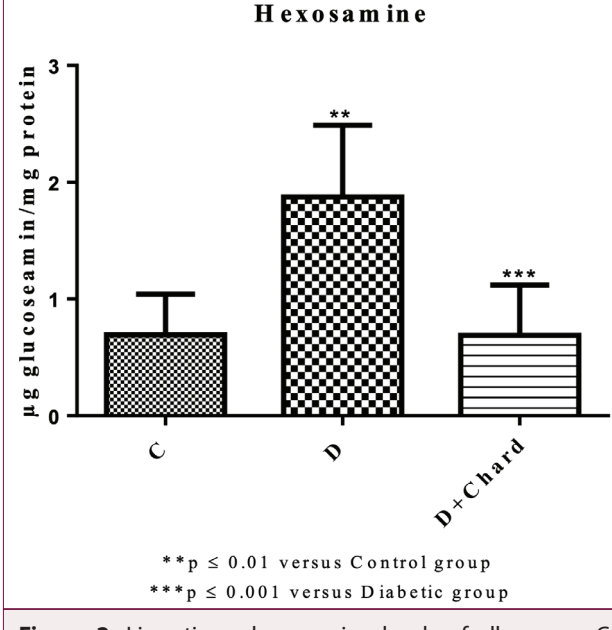

Figure 2. Liver tissue hexosamine levels of all groups. C (Control rats); D (STZ-induced diabetic rats); D+Chard (STZ-induced diabetic rats given chard extract).

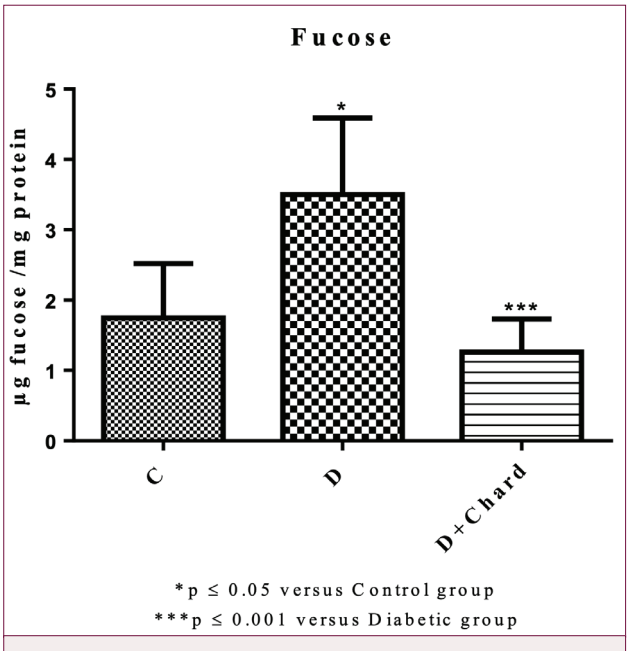

Figure 3. Liver tissue fucose levels of all groups. C (Control rats); D (STZ-induced diabetic rats); D+Chard (STZ-induced diabetic rats given chard extract).

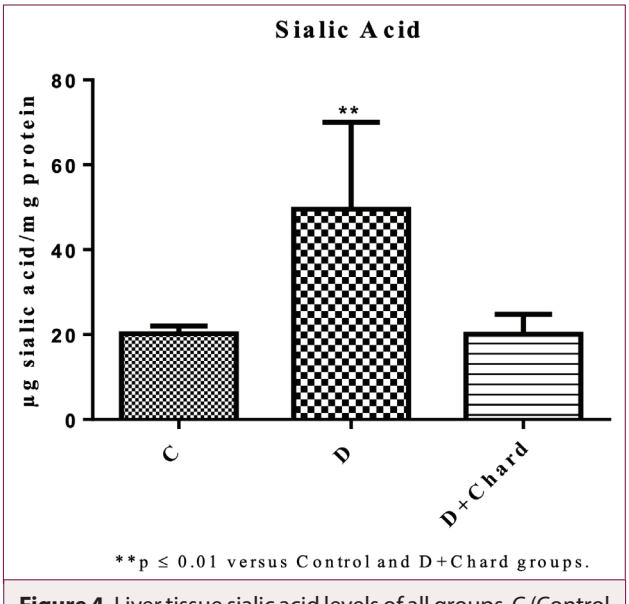

Figure 4. Liver tissue sialic acid levels of all groups. C (Control rats); D (STZ-induced diabetic rats); D+Chard (STZ-induced diabetic rats given chard extract).

The AOPP levels in the liver tissue are presented in Figure 5. In the diabetic group, the level of AOPP was increased ($p \leq 0.001$). The administration of chard reversed AOPP level in the diabetic group ($p \leq 0.001$).

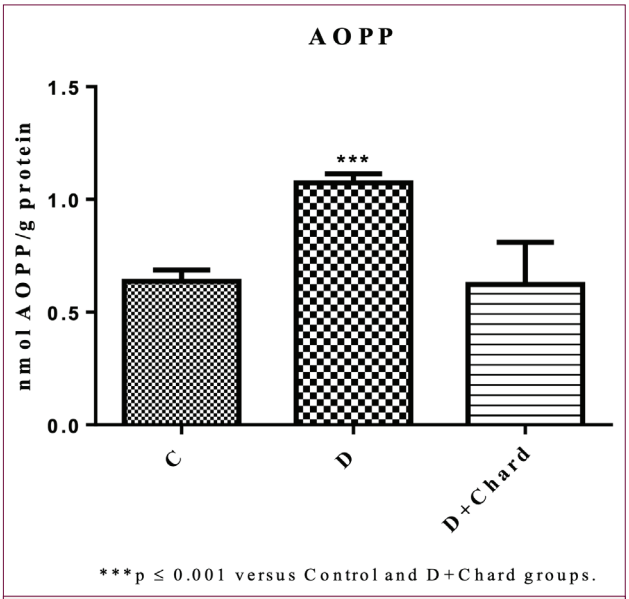

Figure 5. Liver tissue AOPP levels of all groups. C (Control rats); D (STZ-induced diabetic rats); D+Chard (STZ-induced diabetic rats given chard extract).

DISCUSSION

Diabetes mellitus is a chronic disease that can affect many tissues, such as those in the liver, kidney, brain, skin, and lung. Therefore, experimental diabetes models are very important for the understanding and treatment of diabetes. The STZ-induced diabetes model is very similar to human diabetes and is, therefore, often the preferred diabetes model (22).

Throughout history, people have used herbs for medicinal purposes. These medicinal plants have been used to control diabetes and other diseases due to their perceived pharmacological effect. These herbs have low toxicity and have less side effects compared to several conventional drugs. Changes in insulin levels affect glucose and glycogen metabolism in the liver. Therefore, the liver can be an important target tissue in diabetes, and protecting it from the harmful effects of diabetes is paramount (23). The glycoprotein components of the liver are assayed because of their biological importance in diabetes (24). Thus, hexose, hexosamine, fucose, and sialic acid levels were determined in the present study.

Hexosamines are amino sugars in which an amino group replaces a hydroxyl group, the first step of the synthesis of blood and tissues glycoprotein. The level of hexosamines is very important for understanding the harmful effects of diabetes. Several studies have shown that hexose and hexosamine levels are increased in the plasma and tissues of diabetic rats. In our previous study, we reported that the hexose and hexosamines levels in the lungs of diabetic rats were elevated. Treatment with chard extract was seen to reduce hexose and hexosamine levels when compared to the diabetic group (25). In our present study, the chard treatment of diabetic rats significantly decreased liver hexose and hexosamines levels to near-normal levels. This is likely due to the antihyperglycemic effects of chard leaves, which possibly increased insulin secretion.

Fucose is an essential sugar responsible for cell-to-cell communication. Its metabolism is a marker for several diseases, including diabetes mellitus. Haptoglobin, alpha-1-acid glycoprotein, and alpha-1-antitrypsin, which are proteins, are synthesized by the liver and have a potential role in increasing fucose level. The metabolism and synthesis of these proteins may be disrupted in diabetic conditions, thereby causing an increase in the level of fucose. Owing to an increased glucose level and subsequently higher glycation in diabetic states, fucose level might be increased (24). A significant increase in liver fucose levels of diabetic rats was observed in the present study. Chard extract reversed this abnormality (the level of fucose).

Sialic acid is derived from neuraminic acid and can be found in glycoproteins structures. It plays a role in intracellular signalling, protease resistance, cell-cell recognition, conformational stabilization, and protein targeting (26). The sialic acid levels of serum and tissues are seen as a marker in diabetes (28), in which, the amount of sialic acid increases. This increase in insulin-independent tissues may be due to an increase in sialic acid synthesis and enzyme activity of sialyltransferase. In the present study, chard extract treatment decreased the sialic acid levels of diabetic rats.

AOPP are cross-linked proteins containing dityrosine and are safe markers for assessing the oxidative modification of proteins (29). They usually are produced during oxidative stress or by myeloperoxidase in activated neutrophils through the interaction of hypochlorous acid and chloramines. The accumulation of AOPP in tissues plays an important role in the long-term complications of diabetes. Gradinaru et al. stated that AOPP levels are elevated in diabetic patients (30). An increased production of AOPP in the livers of diabetic rats was observed in the present study. Treatment with chard extract significantly reduced the AOPP levels in diabetic rats. This result indicates that chard may be effective in preventing oxidative protein damage by reducing oxidative stress through its flavonoids contents.

The outcome of the present study showed that chard has a beneficial role in diabetic rats' livers by decreasing the levels of glycoprotein metabolites and AOPP as well. The results also demonstrated that consumption of chard for 45 days may stabilise the levels of glycoproteins and AOPP. Therefore, chard can be a potentially effective vegetable for controlling and deescalating the complications of diabetes.

Ethics Committee Approval: Ethical approval was obtained from Marmara University Animal Care and Use Committee (No: 68.2008.mar).

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Expression Levels of B Cell Receptor (BCR) Signaling Pathway Factors are Associated with Conversion from Clinically Isolated Syndrome to Multiple Sclerosis

B Hücre Reseptör (BHR) Sinyal Yolağı Faktörlerinin Ekspresyon Düzeyleri Klinik İzole Sendromdan Multipl Skleroza Dönüşümle İlişkilidir

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ABSTRACT

Objective: We have previously shown altered blood B cells in the first attack blood samples of clinically isolated syndrome (CIS) patients, who converted to multiple sclerosis (MS). Our aim was to investigate levels of B cell receptor (BCR)-associated genes expression in converting CIS (CIS-c) and non-converting CIS patients (CIS-nc) with a 5-year follow-up.

Methods: Seven CIS-c patients, 12 CIS-nc patients and 10 age-sex matched healthy donors were enrolled in this study. RNA was extracted from frozen peripheral blood cells of subjects that were obtained in the first attack using a RNA isolation kit. Expression levels of B-cell adaptor protein with ankyrin repeats 1 (BANK1), B lymphocyte kinase (BLK) and Fc receptor-like protein 2 (FCRL2) genes were evaluated samples by real-time polymerase chain reaction (RT-PCR).

Results: CIS-nc patients showed elevated BANK1, BLK and FCRL2 gene expression levels as compared to CIS-c patients and healthy controls.

Conclusions: Expression levels of the genes that inhibit BCR signaling are increased in CIS-nc, suggesting that inhibiting exaggerated B cell response by optimization of B cell proliferation may prevent the progression of clinically isolated syndrome to MS.

Keywords: Multiple sclerosis, B cell receptor, BANK1, BLK, FCRL2, clinically isolated syndrome

ÖZ

Amaç: Daha önceki çalışmalarımızda, multipl skleroza (MS) dönüşen klinik izole sendrom (KİS) olgularının ilk atakta alınan kan örneklerinde periferik kan B hücre düzeylerinin değiştiğini gösterilmiştir. Bu çalışmada beş yıllık takipte MS'e dönüşen ve dönüşmeyen KİS olgularında B hücre reseptör (BHR) ilişkili genlerin ekspresyon düzeylerinin araştırılması amaçlanmıştır.

Yöntem: Yedi MS'e dönüşen, 12 MS'e dönüşmeyen KİS olgusu ile yaş ve cinsiyet açısından eşleştirilmiş 10 sağlıklı donör çalışmaya dahil edildi. İlk atak döneminde elde edilen donmuş periferik kan mononükleer hücrelerinden (PKMH) RNA izolasyon kiti kullanılarak total RNAlar izole edildi. Örneklerden gerçek zamanlı polimeraz zincir reaksiyonu (RT-PCR) yöntemi ile ankrin tekrarlı B-hücre adaptör proteini (BANK1), B lenfosit kinaz (BLK), Fc reseptör benzeri protein 2 (FCRL2) genlerinin ekspresyonları değerlendirildi.

Bulgular: MS'e dönüşmeyen KİS olgularında BANK1, BLK ve FCRL2 gen ekspresyon düzeyleri MS'e dönüşen KİS olguları ve sağlıklı kontrollere kıyasla artmış olarak saptandı.

Sonuç: BHR sinyalizasyonunu inhibe eden gen ekspresyonlarının MS'e dönüşmeyen olgularda artması, abartılı B hücre yanıtının B hücre proliferasyonunun optimize edilmesi yoluyla baskılanmasının KİS'ten MS'e ilerleyişi engelleyebileceğini düşündürmüştür.

Anahtar Kelimeler: Klinik izole sendrom, multipl skleroz, B hücre reseptörü, BANK1, BLK, FCRL2

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INTRODUCTION

Multiple sclerosis (MS) is a disabling autoimmune central nervous system disease characterized by myelin loss, axonal degeneration that affects mostly young adults (1). Although it has been long implicated that myelin's antigen specific T cells are the main drivers of MS pathology, recent studies showed that B cells also have crucial roles in the disease mechanism (2).

Due to the presence of meningeal B cell follicles in the brains of patients with the secondary progressive form of MS and the effectiveness of drugs targeting B cells, it is perceptible that abnormal activation of B cells may be a part of MS pathogenesis (3). T cell counts as well as the IFN-γ and IL-17 levels are found to be decreased in MS patients after treatment with anti-CD20 monoclonal antibodies, compared to pretreatment levels. This finding suggests that B cells may contribute to MS pathogenesis through an antibody-independent mechanism of action, such as presenting antigens to T cells or regulating cytokine production (4).

Clinically isolated syndrome (CIS) is seen in approximately 85% of MS patients, and about 60% of them convert to clinically definitive MS (CDMS) in 20 years (5). B cell counts and levels of B-cell chemo-attractant CXCL13 are increased in the cerebrospinal fluid (CSF) of CIS patients indicating the presence of prominent B cell activation in the early phase of MS (6). In a previous study, we demonstrated that the total B cell counts in the blood samples of CIS patients, who converted to CDMS (CIS-c) were lower compared to non-converting CIS patients (CIS-nc). On the other hand, CIS-nc patients showed reduced frequencies of plasma cells and memory B, production of which requires B cell receptor (BCR) signaling pathway activation (7).

We previously showed through transcriptome studies that B cell related genes differently expressed between benign and non-benign MS patients. Bioinformatics analysis and clinical correlations suggested that B lymphocyte kinase (BLK), B-cell adaptor protein with ankyrin repeats 1 (BANK1) and Fc receptor-like protein 2 (FCRL2) expression levels could be related with prognosis (Turkoglu et al., unpublished data). BLK is a tyrosine kinase that plays an important role in early B cell development and interacts with BANK1, an adaptor protein that involves in germinal center formation (8,9). FCRL2 is an immunomodulatory protein that inhibits B cell receptor signals and is expressed in transformed B cells (10). Thus, we selected these genes for evaluation of B cell related factors in conversion from CIS to CDMS.

In this study, our aim was to investigate the significance of BCR activity in conversion from CIS to MS through comparative measurement of the peripheral blood expression levels of B cell factors that are involved in BCR signaling pathway activation.

MATERIALS AND METHODS

Subjects

Twelve CIS-nc patients, who did not convert to CDMS in a five-year follow-up, seven CIS-c donors, who converted to CDMS in the same follow-up duration and 10 age-sex matched healthy controls (HC) with no history of prior inflammatory disease were included. CIS was defined as the first demyelinating attack and conversion from CIS to CDMS was determined according to revised McDonald Criteria (11). Patients who received immunosuppressive treatment in the last 3 months or had another coexisting disease, were excluded. Clinical evaluation was performed and the expanded disability status scale (EDSS) scores were determined. Oligoclonal IgG bands (OCB) were evaluated in patient groups.

Patients were followed up every 6 months for five years by the same neurologist and whenever a relapse was suspected

Written informed consent was approved by all participants and the study was approved by Istanbul University, Istanbul Faculty of Medicine, Clinical Research Ethical Committee with the protocol ID of 2012/153937 in 2012.

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (RT-PCR)

Blood samples were collected 4 weeks after the first attack, while patients were not using any medications including immunosuppressive and immunomodulating drugs. Peripheral blood mononuclear cells (PBMCs) were isolated from blood and cryopreserved in storage solution until use (density gradient centrifugation).

The total RNA was extracted from the frozen PBMCs of subjects using a RNA isolation kit (Qiagen, RNeasy Plus Mini Kit, Hilden, Germany) according to the manufacturer's instructions. The cDNA was synthesized by using a SCRIPT cDNA Synthesis Kit (Jena Bioscience, GmbH, Frankfurt, Germany) as per manufacturer's recommendations in T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., CA, USA) as followed cycle conditions: 10 minutes at 42°C, 60 minutes at 50°C, and 10 minutes at 70°C. The cDNA samples were diluted as the final concentration would be 200 ng/µl.

The RT-PCR assay was performed on a Lightcycler® 480 II Instrument (Roche Applied Science, USA) by using LightCycler® 480 SYBR Green I Master. The optimal program of the RT-PCR includes an initial pre-incubation at 95°C for 8 minutes, followed by 45 cycles of amplification at 95°C for 15 s, primer-dependent °C (57-63) for 60 s, 72°C for 20 s. A melting step was performed at the end of the amplification using the following cycling parameters: 95°C for 5 s, 65°C for 60 seconds and continuous at 95°C. Primers designated for BANK1 (F: 5′-GTTCAGACCCCGCACATATT-3′ and R: 5′-CCTTCCCCTTCCATTTCATT-3′), BLK (F: 5′-TAGATCACAGGGTCGGAAGG-3′and R: 5′-GGCAGCGGATCTTATAGTGC-3′), FCRL2 (F: 5′-CTCTGGGGACTGTTTGGTGT-3′ and R: 5′-GGTTGGGCTTGAATAGGTGA-3′) and as a housekeeping gene GAPDH (F: 5′-CCATCAATGACCCCTTCATT-3′) and R: 5′-TTGACGGTGCCATGGAATTT-3′). The relative mRNA expression

was measured using the simplified comparative threshold cycle delta, cycle threshold method.

A melting curve analysis was obtained at the end of the cycles and cycle threshold values were calculated from the system software.

Statistical analyses

SPSS 20 software was utilized for statistical analysis and graphs were performed using GraphPad Prism software. The significance levels of clinical and demographical findings and expression levels of the genes were calculated using the Kruskal-Wallis or Mann–Whitney U tests, as required. Categorical parameters were compared by using the Chi-square test. Correlation analysis was done with Spearman's test.

RESULTS

Clinical and demographic findings

There was no difference in EDSS scores, age, or gender among the study groups. Since CIS-nc patients displayed only one attack in 5 years, CIS-c patients had inadvertently significantly higher total and annual number of attacks than CIS-nc patients. Although the prevalence of OCB positivity and the number of MS lesions were statistically comparable, CIS-c patients showed trends towards displaying a higher ratio of OCB positivity and brain lesions. Clinical and demographical characteristics of participants were demonstrated in Table 1.

B cell genes' expression levels

CIS-nc patients showed trends towards exhibiting elevated expression levels of all three B cell-associated genes. BANK1 expression (p=0.074) was significantly higher in CIS-nc patients than HC (p=0.040) and showed trend significance compared to CIS-c patients (p=0.097). BLK gene expression levels were significantly higher (p=0.024) in CIS-nc patients in comparison to CIS-c patients (p=0.013) and HC (p=0.005). CIS-nc patients also showed significantly higher (p=0.018) FCRL2 expression levels than HC (p=0.003) but not CIS-c patients (p>0.1). There were no correlations between gene expression levels and clinical and demographical parameters of CIS-c and CIS-nc patients (data not shown).

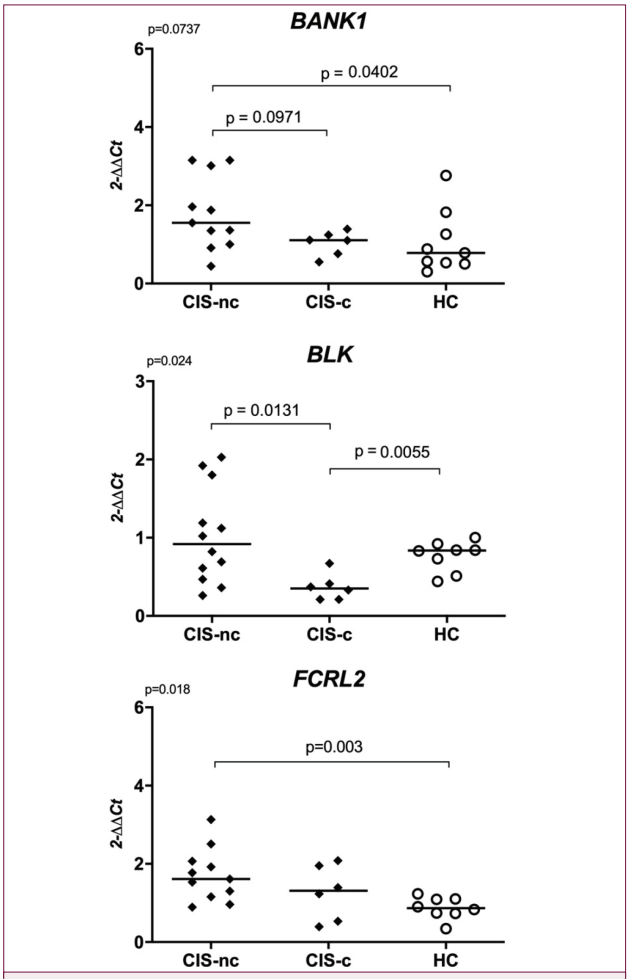

Figure 1. Comparison of relative expression levels of B cell specific genes in study groups. Short horizontal lines indicate mean values. p value on the upper left corner is obtained by Kruskal-Wallis test, whereas p value over the long horizontal line is obtained by two-group comparisons using Mann-Whitney U test. CIS-nc: Non-converting clinically isolated syndrome, CIS-c: converting clinically isolated syndrome, HC: Healthy control. Note that only significant p values (p<0.05) and p values with trend significance (p<0.1) were denoted.

Table 1. Clinical and demographical characteristics of healthy controls and CIS patients at the 5th year of follow-up.

	CIS-nc (n=12)	CIS-c (n=7)	HC (n=10)	p value
Gender (Female/Male)	9/4	5/2	7/3	0.994
Age (mean±SD)	39.83±3.0	38.14±2.58	41.70±4.27	0.315
EDSS (mean±SD)	1.42±0.56	1.29±0.27	NA	0.967
Total number of attacks (mean±SD)	1.00±0.00	2.14±0.82	NA	**0.008**
Annual number of attacks (mean±SD)	0.20±0.00	0.68±0.44	NA	**0.005**
OCB (pattern 2 or 3) positive/negative	7/5	6/1	NA	0.215
Number of MS lesions on MRI	3.42±3.75	8.86±9.26	NA	0.173

CIS-nc: Non-converting clinically isolated syndrome; CIS-c: Converting clinically isolated syndrome; HC: Healthy control; EDSS: Expanded disability status scale scores; OCB: Oligoclonal band; MRI: Magnetic resonance imaging; NA: Not applicable; SD: Standard deviation. Significant p values were denoted with bold characters

DISCUSSION

In our previous study, we showed that the most significant peripheral blood B cell subset differences between CIS-c and CIS-nc were observed in effector B cells (7). In this study, we investigated the expression of the genes, which regulate BCR signaling, and found that CIS patients with increased baseline expression levels of FCRL2, BLK, and BANK1 were less likely to develop CDMS in a five-year follow-up duration. Our results show that peripheral blood expression levels of certain genes may predict prognosis after a single demyelinating attack. To our knowledge, this type of association between these genes and MS has been shown for the first time.

The next question was whether altered expression of these genes prevented MS conversion through suppression of BCR and thus decreased production of effector memory phenotype of B cells and plasma cells. B cell antigen receptor mediated signals are crucial for survival, proliferation, and development of B cells and regulated by activator and inhibitor signals. Defects of BCR signaling can cause a predisposition to autoimmune disorders (12). BANK1 is an adaptor protein recently found to be related to the BCR-associated calcium homeostasis and is expressed in immature, mature, and marginal zone B cells but could not be detected in $CD3^+$ T cells, dendritic cells, and macrophages (9). It has been shown that BANK1 attenuates CD40 mediated B cell activation thereby preventing exaggerated B cell responses (13).

Phosphorylation of BANK1 protein after BCR stimulation is provided by its interaction with phospholipase C gamma 2 and BLK, a kinase belonging to the Scr family and primarily expressed in B cells and thymocytes (14,15). Although the role of the BLK protein in humans has not been fully elucidated, its association with autoinflammatory/autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) suggests that alteration in BLK expression may cause changes in tolerance mechanisms in B cells (16). Since most of the disease-related BLK haplotypes are correlated with a decrease in BLK expression, lower levels of BLK confer risk for autoimmunity (17).

Another regulator of the BCR signaling pathway is FCRL2, a new member of the immunoglobulin superfamily dominantly expressed by memory B cells, mature B cells, germinal center B cells; slightly expressed in immature bone marrow and plasma cells and contributes to the autoimmune pathogenesis (18). It has been shown to suppress the BCR signaling through the activation of "Src homology region 2 domain-containing phosphatase-1" (SHP-1) (19). Mutations in the FCRL2 genes were found to be associated with SLE, insulin-dependent diabetes, and have indicated an immunomodulatory role of FCRL2 (20). Consistent with our findings, FCRL was found to be downregulated in PBMCs of MS patients with high neurodegeneration signs on magnetic resonance imaging (MRI) (21). In view of these findings, our results imply that a compensatory increase of BANK1, BLK and FCRL2 expressions might be inhibiting BCR signals and thus preventing conversion of CIS to CDMS through suppression of exaggerated B cell responses.

Among the limitations of the study, we could not isolate B cells from peripheral blood due to the amount of the blood samples from patients, since our B cell immunophenotyping results were not different in terms of B cell ratios between patients and healthy subjects (11.87% for CIS vs. 8.37% for RRMS vs. 8.37 for HC) (7), we evaluated gene expressions in PBMC samples by RT-PCR. The other limitation of our study was a low sample size due to the drop-out of patients who did not regularly attempt follow-up visits.

CONCLUSION

Our findings suggest that B cells may play an essential function in the conversion of CIS to CDMS, and the increase in the expression of B cell regulating genes might prevent this conversion. Meanwhile, evaluating the expression of these genes for the disease in isolated B cells could provide a more specific inference. Also, investigation of potential genetic variants of these genes in MS is warranted. BCR inhibition might be a potential therapeutic target to prevent CIS-MS conversion.

Ethics Committee Approval: The study was approved by the Istanbul Faculty of Medicine Clinical Research Ethical Committee with the protocol ID of 2012/153937.

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Choice of Implant Surface-Feature May Affect the Viability of the Adherent Cells

İmplant Yüzey Özelliği Seçimi Adherent Hücrelerin Canlılığını Etkileyebilir

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ABSTRACT

Objective: *In vitro* evaluation of implant materials' effects on cell adhesion and viability can provide useful information for predicting implant biocompatibility. Therefore by using a simple and inexpensive method, it was aimed to investigate whether different implant surface-features might have distinct effects on the viability and adherence of the cells.

Material and Methods: Different dental implant surfaces (anodized (AN), blasted wrinkled (BW), grit/acid etched (GA), and hydroxylapatite sprayed (HB)) were tested for their possible effects on adhesion and viability of the adherent human osteoblast cells by using an agar-based *in vitro* technique. Viability of the cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue staining.

Results: The rate of cell adhesion did not seem to be significantly affected by the differences in surface features of dental implants (AN:78.21±0.52; BW:78.22±0.48; GA:78.44±0.85; HB:77.26±0.96). The surface features of the dental implants had an impact on the viability of the attached cells on the implants. Viability of the attached cells was significantly higher on AN, BW, GA surfaces when compared to the HB surface (AN: 72.28±6.04, BW: 67.02±3.47, GA: 85.82±5.05, and HB: 27.98±10.47).

Conclusions: *In vitro* findings suggests that AN, BW, GA surfaces may provide a better platform than HB surfaces to maintain the viability of bound cells.

Keywords: Biocompatible surfaces, irregular surfaces, cell adhesion potential, cell culture, dental implants

ÖZ

Amaç: İmplant materyallerinin hücre canlılığı ve hücre adezyonu üzerindeki *in vitro* etkilerinin değerlendirilmesi, implantların biyo-uyumluluklarının tahmin edilmesi yönünde değerli bilgiler sağlayabilir. Bu nedenle, basit ve maliyetli olmayan bir yöntem kullanılarak, farklı yüzey özelliklerine sahip implantların hücre canlılığı ve adezyonunu etkileyip etkilemeyeceğinin araştırılması amaçlanmıştır.

Gereç ve Yöntemler: Farklı diş implant yüzeylerinin (anotlanmış (AN), patlatılmış buruşuk (BW), kum/asitle aşındırılmış (GA), püskürtülmüş hidroksi apatit yüzey (HB)) yapışma ve canlılık üzerindeki etkileri, adherent insan osteoblast hücre soyunda agar bazlı bir *in vitro* teknik kullanılarak karşılaştırılmıştır. Hücre canlılıklarının belirlenmesinde, 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromid (MTT) ve tripan mavisi boyama teknikleri uygulanmıştır.

Bulgular: Hücre adezyonunun, dental implantların yüzey özelliklerindeki farklılıklardan önemli ölçüde etkilenmediği görülmüştür (AN: 78,21±0,52; BW: 78,22±0,48; GA: 78,44±0,85; HB: 77,26±0,96). Ancak, diş implantlarının yüzey özelliklerinin, implantlara tutunan hücrelerin canlılığı üzerinde etkisi olduğu tespit edilmiştir. Tutunan hücrelerin canlılığının, HB yüzeyine kıyasla AN, BW, GA yüzeylerinde anlamlı olarak daha yüksek olduğu bulunmuştur (AN: 72,28±6,04, BW: 67,02±3,47, GA: 85,82±5,05 ve HB: 27,98±10,47).

Sonuçlar: Çalışma kapsamında yapılan *in vitro* deneylerden elde edilen bulgular; AN, BW, GA yüzeylerinin adherent hücrelerin canlılığını korumaları için HB yüzeylerinden daha iyi bir platform sağlayabileceğini göstermektedir.

Anahtar Kelimeler: Biyouyumlu yüzeyler, düzensiz yüzeyler, hücre adezyon potansiyeli, hücre kültürü, dental implantlar

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INTRODUCTION

Implants are useful instruments used in a variety of clinical settings, including orthopedics and dentistry. Technology and research on implantation materials have greatly expanded in recent years. For example, titanium dental implants are routinely used for the treatment of tooth loss. The success of the operation in part depends on the reaction of the tissue to the implant. The reaction of the tissue to the implants can be divided into two stages: formation of fibrous soft tissue capsules around the implant and osseointegration (1). The fibrous tissue capsule helps biomechanical fixation, therefore the absence of this structure can lead to dental implant failure. The surface property of the biomaterial can determine the success of the osseointegration process. Various modifications can be made on dental implant surfaces to improve osseointegration.

Cell adhesion is critical for the implant's effective incorporation into bone tissue. For this reason, the use of a biocompatible surface that would not have a negative effect on proliferation, adhesion, and phenotype expression of osteoblasts is recommended (2).

The roughness and physical changes made on the biomaterial surface are known to affect the response of tissues and cells to implantation (3). The two most commonly used methods to roughen the surfaces of dental implants include etching and coating techniques. Sand-blasting and pickling methods are common practices for etching (3). The most commonly used techniques in the coating method are bioactive calcium phosphate ceramic coatings and titanium plasma spray coatings (4). Titanium plasma-spraying (TPS) and hydroxyapatite (HA) coatings are frequently used as coating materials. Microporous or nanoporous surfaces can also be produced with anodization of titanium at the high electric current or another way to roughen titanium dental implants is to use heavy acids (e.g., HF, H_2SO_4, HNO_3, H_3PO_4) (1).

There are several different experimental methods to evaluate adhesion competence. For example, the "washing" technique makes identifying adherent cells with a short lifespan simple, allowing cells detached from the implant's surface to be counted (5). Also, cell separation technology, micropipette suction technology, the measurement method of force spectroscopy, force probe for biofilms, optical tweezers, rotating disk method are available techniques that can be used (6). These methods, however, are not readily available in every laboratory, and they may not be successful in determining the rate of cell proliferation and adhesion on irregular surfaces like implant grooves.

Evaluation of the *in vitro* effects of implant materials offers many advantages on the prediction of the success and biocompatibility of the implants. In this respect, it is important to investigate the effects of materials on cell viability as well as the adherence of the cells. Inexpensive, simple, rapid, and accurate techniques can provide convenience for researchers in terms of the pre-evaluation of the materials. In this study, the effects of different implant surfaces were tested on the adhesion and proliferation rate of human osteoblasts. A modified version of the wash-of process, which was recently defined, was used for this purpose (7). Using this inexpensive and simple *in vitro* technique, the impact of dental implants with the anodized surface (AN), blasted wrinkled surface (BW), grit-blasted/acid-etched surface (GA), or hydroxyl apatite-blasted surface (HB) features were compared.

MATERIALS AND METHODS

Cell culture

The human osteoblast (HOB) cell line (406-05F, Sigma-Aldrich) was cultivated in high glucose Dulbecco's Modified Eagle's medium (5546 DMEM-Sigma-Aldrich) and 10% fetal bovine serum (heat-inactivated., S1810-500, Biowest), 1% 2mM l-glutamine (Biological Industries, BI03-020-1B), and P/S (50 U/mL streptomycin and 50 g/mL penicillin; 03-031-1B, Biological Industries) was added to the mix (complete medium). $1.5x10^6$ cells were seeded into 10cm plates. For 72 hours, at 37°C, the plates were incubated with 5% CO_2 before being split. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue assays were performed using trypsinized cells seeded in 96-well flat-bottom culture plates.

Preparation of implants and the internal control

Agar-coated, chromium-plated metal screws with similar dimensions (4x15 mm) were used as an internal control. Agar-coated screws served as a platform to eliminate the non-specific binding artifacts that may arise due to inadequate rinsing or as a result of clumps that got stuck between the grooves. All the dental implants were compared against this internal control. Information about the implants with different surface features used in the study is given in Table 1. Dental

Table 1. Dental implants with different surface features.

Surface feature	Abbreviation	Brand model	Dimensions	Lot number
Anodized surface implants	AN	Nobel Biocare™ NobelParallel Conical Connection RP	4.3x15.0 mm	12103733
Hydroxyl apatite-blasted surface implants	HB	Arrow™ -C4014S	4.0x14.0mm	W5H27BY
Blasted wrinkled surface implants (Sandblasted)	BW	Dentegris™ 45014-SL SL-Tapered	4.5x14.5 mm	17042708
Grit-blasted/acid-etched surface dental implants (Ca and P low impregnation)	GA	Ossean-Intra Lock™	4.0x15.0 mm	BM110

implants were provided as gamma-ray sterilized ready-to-use implants. Sterilization of the internal control metal screws was done with an autoclave. In this study, 12 implants were used. Each time new implants and screws were used for experiments. Representative images of dental implants are shown in Figure 1a. As explained previously (7, 8), the internal control screws were coated with 0.2% agar (Multicell- 800-010-LG) to simulate conditions where cells cannot attach. As shown in Figure 1b, coating the metal screws with agar was performed by placing sterile screws (6-well) into the wells. 0.2% agar solution was prepared in phosphate-buffered saline (PBS) and 3 mL of agar solution was poured into the well and stored for 3 hours at room temperature. The wells were washed with 2 mL of 1×PBS, as described previously. (7).

Agar petri dishes preparation and seeding cells

The implants and screws at the bottom of the 10 cm petri dishes were stabilized with a drop of molten candle, as shown in Figure 1c. The implants and the internal control screw (with agar coating) were placed side by side in a single 10 mm petri dish. The implants and the internal control screw were then partially immersed in liquid agar (1.5%), which was then allowed to solidify. Since cells can't bind to agar's surface, semi-submerged implants and agar-coated screws were the only places for cells to attach (7). Thus, non-specific binding artifacts may arise due to inadequate rinsing or as a result of clumps getting stuck between the grooves. Following that, onto the agar plate, 6 mL of complete medium containing $4x10^6$ cells/mL were poured, as shown in Figure 1c, unless the medium was fully covered the implants and the internal control screw. Since the cells were suspended in the medium before being inserted into the plate, it was expected that all exposed agar surfaces, implants, and screws would receive approximately equal numbers of cells. Although the implants and the agar-coated internal control screw were both located in the same plate, a side-by-side comparison was possible (7).

Evaluation of the attachment performance of cells

Following the addition of cell suspensions, the petri dishes were kept in the incubator for 24 hours (37°C, 5% CO_2) to enable the cells to bind along. Non-adherent cells were then washed away by rinsing the petri dish three times with washing buffer (PBS, pH 7.4) and discarding the washing buffer, leaving only the securely attached cells (7). After washing, 6 mL of medium was added and petri dishes were placed in the incubator and incubated for 48 hours (7).

The number of detached cells' determination (DC values)

The petri dishes were washed twice with 1×PBS buffer at the end of the 48-hour incubation period. During this period, wash buffer was collected in centrifuge tubes (and not discarded), and the detached cells (DC) were pelleted by centrifugation. Using trypan blue staining, the number of DC was determined. (7).

The number of adherent cells' determination (AC values)

Next, using a sterile scalpel (SPL-90020, blade width 13 mm, length 230 mm,1/sleeve) the agar around the implants and the internal control screw were cut. Then, the agar blocks still containing the buried implants or the internal control screw were transferred into the wells of a 6-well plate containing 1200 µL of trypsin-EDTA and incubate in an incubator at 37°C for 5 minutes. Variations in the size of the cut agar block are unlikely to impact the calculation since the cells cannot bind to the agar

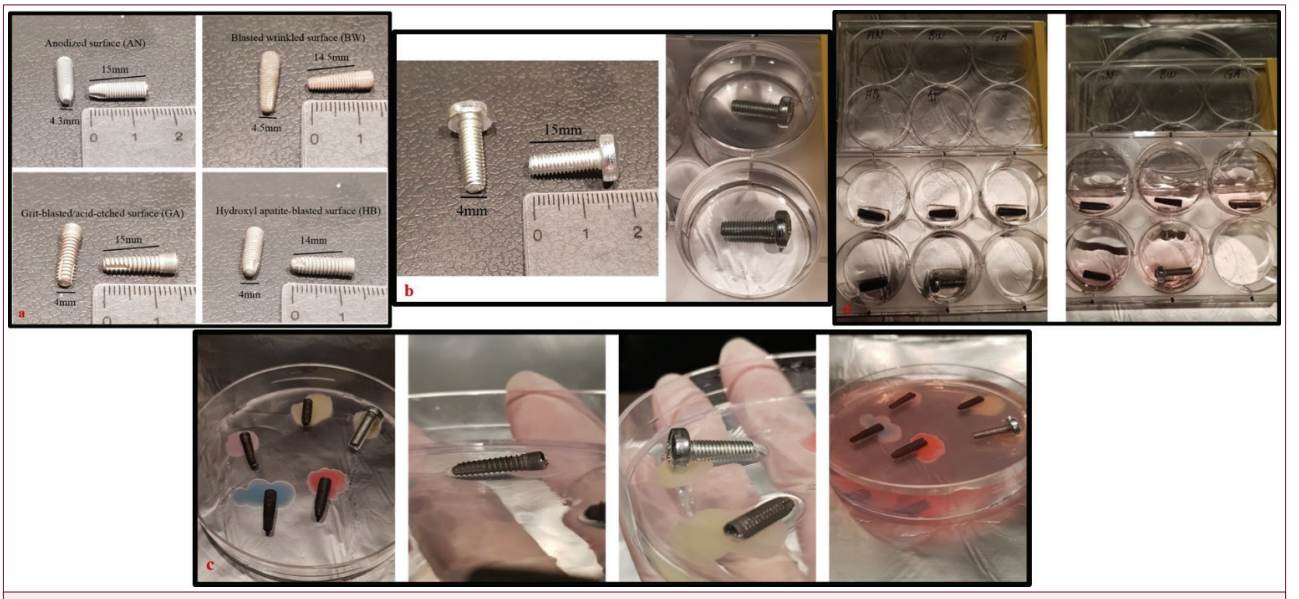

Figure 1. Representative images from different stages of the technique. a. Images of different surfaced dental implants, **b.** Internal control (chrome-plated screws), **c.** A drop of a molten candle was used to secure implants and screws, **d.** Detaching cells with trypsinization from implants and the internal control in 6-well plates.

surface. Then, as shown in Figure 1d, 2400µL of medium was added into the wells to inactivate the trypsin. Centrifugation was used to collect the cells. Then the cell pellet was resuspended in 200µL of medium. Trypan blue staining of 15µL of this cell suspension was used to assess the number of adherent cells (AC). MTT assay was performed with the remaining 185µL of cell suspension (7).

The ratio of adherent cells' determination (RCA)

The ratio of adherent cells (RCA) was determined using the formula: RCA%= AC/ (AC+DC) *100 (7).

Data analysis and relative RCA values calculation

As a reference value, the RCA value obtained from the internal control screw coated with agar ($RCA_{reference}$) was used. The delta RCA value was then calculated by subtracting the reference RCA value from the RCA values obtained from various dental implants using the formula below: $\Delta RCA_X\%= (RCA_X\%-RCA_{reference}\%)$ (7).

Trypan blue staining

To obtain the cell viability, 1:1 mixture of trypan blue (0.4%) prepared in PBS and 15µL of cell suspension was used. The number of the stained (dead) cells and unstained cells were determined within 5-10 minutes with a hemocytometer (9).

MTT assay

MTT test, *in vitro* cytotoxicity assays (cell viability assays), was carried out according to Scudiero's (10) instructions. The 96-well plate was modified from the 24-well plate format. In summary, trypsinized cell pellets were suspended in 200µL complete medium and seeded into 96-well plates. For 18 hours (37°C, 5% CO_2), the cells were incubated. The wells were then gently rinsed with 1xPBS. Every well obtained 200µL of MTT (3580GR001, CAS: 298-93-1., Biofroxx., Lot: 5A13FBF0,) reagent (0.5mg/mL) prepared in DMEM. MTT solution was discarded after 4 hours of incubation at dark (37°C, 5% CO_2), and formazan crystals were solubilized with DMSO (200µL/well). A spectrophotometer was used to record the absorbance at 540nm.

Determining the viability of the adherent cells (vAC)

Absorbance obtained from the MTT assay was used for comparison of the relative changes in the viability of the adherent cells. The mean absorbance value for the internal control (agar-coated screws) was accepted as 100% and used as the reference value (vACreference%). The relative changes were calculated with the following formula: $\Delta vAC_x\%= (vAC_x\%-vAC_{reference}\%)$ (7).

Statistical analysis

GraphPad Prism version 5.0 (San Diego, CA, USA) statistical analysis software was used. Tukey's test was used to make multiple comparisons. It was considered that $p<0.05$ was statistically significant. Analysis of variance was used to examine the groups' viability and adherence indexes. The results were expressed as averages from at least three different experiments for statistical analysis.

RESULTS

Baseline settings for evaluation of cell adherence

When compared to dental implants with different surface features, the data obtained from the agar-coated internal control screws clearly showed that HOB cell attachment on the agar-coated internal control screws was limited (Table 2). As a reference value, the mean RCA value obtained from the agar-coated screws ($RCA_{reference}$%: 39.46) was used. Table 2 shows the RCA values obtained from three separate experiments.

Table 2. RCA% values obtained from three independent experiments.

RCA% VALUES FOR DENTAL IMPLANTS WITH DIFFERENT SURFACE FEATURES				INTERNAL CONTROL
RCA_{AN} %	**RCA_{BW} %**	**RCA_{GA} %**	**RCA_{HB} %**	**$RCA_{reference}$ %**
78.22	78.75	77.48	77.29	39.13
78.70	78.00	79.10	76.30	36.19
77.67	77.86	78.75	78.22	43.06
78.21$_{avg.}$ **(n=3)**	**78.22** $_{avg.}$ (n=3)	**78.44** $_{avg.}$ (n=3)	**77.26** $_{avg.}$ (n=3)	**39.46** $_{avg.}$ (n=3)

RCA%= AC/ (AC+DC) *100, RCA: Ratio of adherent cells' determination, AC: Adherent cells, DC: Detached cells, AN: Anodized surface, BW: Blasted wrinkled surface, GA: Grit-blasted/acid-etched surface, HB: Hydroxyl apatite-blasted surface.

Relative changes in cell adhesion as a result of various surface materials

Relative changes in cell adherence (ΔRCA% values) were calculated as explained under the material and methods section. As shown in Table 3, a comparison of the relative changes in cell

Table 3: Relative changes in cell adherence (ΔRCA% values).

Implants	RCA_X%	$RCA_{reference}$%	ΔRCA_X%
AN	78.21	39.46	**38.76**
BW	78.22	39.46	**38.76**
GA	78.44	39.46	**38.98**
HB	77.26	39.46	**37.80**

The mean reference value for RCA% was 39.46. AN: Aanodized surface, BW: Blasted wrinkled surface, GA: Grit-blasted/acid-etched surface, HB: Hydroxyl apatite-blasted surface

adherence did not reveal a statistically significant difference between the tested surface materials.

Implant surface features affect the viability of the adherent cells *in vitro*

According to the material and methods section, relative changes in the viability of the attached cells (ΔvAC%) were calculated. As shown in Table 4, the mean viability of the attached cells (vAC%) was significantly improved when dental implants were used instead of the agar-coated internal control.

Table 4. Mean changes in the viability of the attached cells.

	vAC%		$vAC_{reference}$%	ΔvAC%
AN	**172.3**	**-**	**100**	**= 72.3**
BW	**167**	**-**	**100**	**= 67**
GA	**185.8**	**-**	**100**	**= 85.8**
HB	**128**	**-**	**100**	**= 28**

Interestingly, as shown in Table 4 and Figure 2, among dental implants with different surface features, significant differences in the viability of the attached cells were detected. When compared with the HB surface, implants with AN surface improved viability by 2.58 fold ($\Delta vAC_{AN}\%$ / $\Delta vAC_{HB}\%$), BW surface improved viability by 2.39 fold ($\Delta vAC_{BW}\%$ / $\Delta vAC_{HB}\%$) and GA surface improved viability by 3.06 fold ($\Delta vAC_{GA}\%$ / $\Delta vAC_{HB}\%$). The lowest viability rate was detected in HB surfaced dental implants. Compared to all the other three implants, the vitality rate was found to be significantly decreased. Furthermore, the effect of BW and GA surfaced dental implants on the viability of the attached cells were significantly different ($p<0.0001$; n=3).

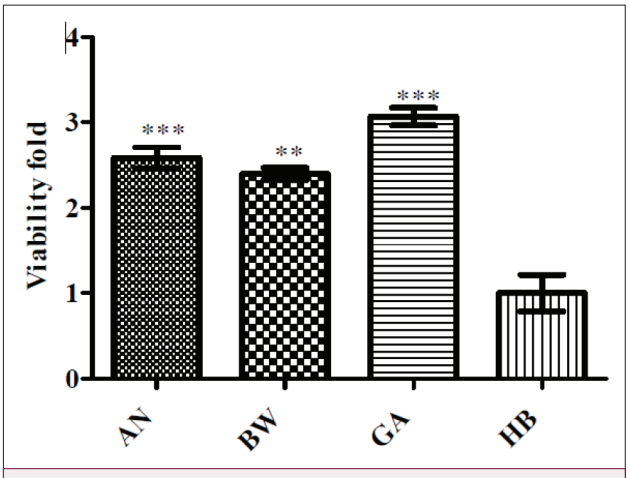

Figure 2. Implant surface features affect the viability of the attached cells.

DISCUSSION

Surface topography and the physicochemical structure of the surface materials are important factors in determining the relation between the implanted biomaterial and the bone (11, 12). Surface features and the geometric design of the implant structures play an important role in the success of dental implants (3). Studies have shown that modification of surface properties (e.g., AN, BW, GA, HB surfaces), can improve osteointegration. The acid etching technique, for example, has been shown to increase the surface area of implants (13). Osteoblasts contact neighboring cells and their environment through their cytoplasmic extensions. Osteointegration involves biomechanical processes where a firm connection between the bone and the implant is formed (14). Osteoprogenitor cells migrate to the implant site and differentiate into osteoblasts and provide bone formation (15, 16). Providing suitable surfaces for attachment of the cells and sustaining the viability of the attached cells is crucial for a successful implant operation. Therefore, the question has arisen whether the different surface features of the implants will affect the cell efficiency and/or attachment viability of the attached cells. To address this question, the effects of different dental implant surfaces (AN, BW, GA, HB) on the adhesion and viability of the attached cells were compared. The effects of the surface properties of dental implants on cell behavior had previously been studied by other groups using model systems (such as titanium discs) to simulate the surface of the implants (17–19). The titanium surface treatment, according to Arcelli et al. (20), had a direct genetic effect on osteoblasts, inducing multiple bone-related genes. On the other hand, Kieswetter et al. (21) found that cell proliferation was inversely related to surface roughness. Similarly, Lauer et al. (22) did not report any differences in osteoblast adhesion and growth on different textured titanium surfaces. In this study, the osteoblast cells were used to determine the *in vitro* effects of these implants. A modified version of the washing technique, recently defined by Gundogan (7), was used to see how different surface features affected cell attachment and viability. In this context, the impact of dental implants with the AN, BW, GA, or HB surfaces features were investigated. When compared to the internal control group, cell attachment rate was significantly improved on the modified surfaces. However, there was no statistically significant difference in the rates of cell attachment when AN, BW, GA, or HB surfaces were compared (Table 3). Interestingly, it has been shown that surface features of the dental implants can affect the viability of the attached cells on the implants. Cell viability was significantly enhanced on the AN, BW, GA surfaces when compared to the HB surface (Table 4 and Figure 2).

Other researchers have examined the physiological conditions of cells using different methods for a detailed and comprehensive investigation. Conserva et al. (23) evaluated osteoblast adhesion and proliferation with qualitative scanning electron microscopy (SEM) analysis. According to Conserva et al. study (23), the SaOS-2 cells spread more rapidly on sandblasted surfaces. They have shown that cells had proliferated

and exhibited a flat morphology after 24 hours of growth. They revealed that the most significant cell flattening was on the grit-blasted/acid-etched surface. Also, they indicated that the grit-blasted/acid-etched surface showed a greater number of SaOS-2 and MSC cells than the sandblasted surface at both 3rd and 7th days of growth, with a significant ($p<0.05$) difference between the two surfaces (23). Contrary to the technique applied in this study, they did not stabilize the implants in the study of Conserva et al. (23) and achieved results with the base of cell lysis by not separating the cells from the implant surfaces. The implants were submerged in the cell suspension throughout the incubation wells at the same time. The implants were gently removed from plates after the incubation time and washed. The findings of Conserva et al., (23), are consistent with the study's adherence and viability results. The GA sample was also superior in this study compared to the BW sample. Both dual acid-etched and alumina-blasted surfaced (Intra-Lock) and P and Ca low impregnation (Ossean-Intra-Lock) surfaced implants provide a suitable substrate for SaOS-2 human osteoblast adhesion and development, according to Bucci-Sabattini et al. (24). Per the Bucci-Sabattini et al. (24), the MTT test for the quantitative proliferation of SaOS-2 revealed a similar proliferation rate on both surfaces with no significant difference. Quantification of alkaline phosphatase (ALP) specific activity in the SaOS-2 cell line, on the other hand, was substantially higher for the Ossean surface at all times (24). Although it is not the same cell line, Bucci-Sabattini et al.'s study (24) also support the findings of this research.

Data from previous studies (*in vitro* or *in vivo*) also suggest that GA surfaces may exhibit superior features. For example, Sammons et al. (25) showed that, compared with plasma-sprayed, acid-etched, grit-blasted, anodized, and machined surfaces the combined surface of grit blasting and acid etching induce a substantial increase in cell spreading rates. An *in vivo* test on dogs found that, compared with titanium plasma-sprayed surfaces, implants with grit-blasted and acid-etched surfaces can be immediately placed into the damaged periodontal area, and their performance is slightly better (26). An experimental study again in dogs evaluated the grit-blasted/acid-etched bio-ceramic and dual acid-etched implant surfaces histomorphometrically (27). When comparing the grit-blasted/acid-etched bio-ceramic implant surface to the dual acid-etched implant surface, the grit-blasted/acid-etched bio-ceramic implant surface had increasing rates of the bone organization along the perimeter, according to Marin et al. (27).

Bone cell interactions with smooth titanium, titanium plasma-sprayed, titanium dioxide-blasted, and hydroxyapatite plasma-sprayed implants were compared by Lumbikanonda & Sammons (28). The suspended neonatal rat osteoblasts lasted for 20 minutes, and the attached cells were classified with SEM according to the stage of attachment (28). Lumbikanonda & Sammons (28) revealed that the cells spread the fastest on titanium plasma-sprayed implants. It was shown that the completely dispersed cells on the smooth titanium implant adhered tightly to the surface, while on the titanium dioxide sandblasted surface, they did not adapt to surface irregularities. In contrast to the hydroxyapatite-coated implants, cells adhere closely only to smooth areas (28). In line with the results of this study, the cell viability was significantly higher on the other surfaces compared to the HB surface.

In agreement with the previously published data, the *in vitro* findings of this study implies that AN, BW, GA surfaces may present a more suitable platform to maintain the viability of bound cells when compared with the HB surfaces. The study findings also show that these *in vitro* evaluations can be performed using an inexpensive and simple agar-based method.

Although reproducible and solid data have been provided to support the findings, insight into the underlying molecular mechanisms and pathways has not be provided due to the lack of molecular techniques. Future studies may be designed to investigate the genes or proteins which may be involved in this differential response of HOB cells to different surface materials.

CONCLUSION

The differential features of commercially available dental implants may affect the viability of the attached cells rather than the rate of cell attachment. The results related to cell viability or adherence can be obtained accurately with an inexpensive and simple agar-based method. It has been showed that AN, BW, GA surfaces can provide a better platform than HB surfaces to maintain the viability of the attached cells.

Ethics Committee Approval: Since this study is a cell line study, ethics committee approval has not been obtained.

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Electronic Learning in Undergraduate Medical Education

Mezuniyet Öncesi Tıp Eğitiminde Elektronik Öğrenme

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ABSTRACT

Objective: This study was designed to assess the impact of electronic education on students' learning levels and to compare the video-based electronic learning with text-based learning among medical faculty students.

Materials and Methods: This study was conducted as a single center, cross-sectional study. All of the volunteers were fourth year students of the medical faculty. All students were randomly divided into two groups. Each group had 100 students. In group 1, all students were educated by video-based electronic learning about wound healing, and in group 2, all students were educated by standard lectures from textbook. Both groups were evaluated according to their correct response rates.

Results: The video-based learning increased the correct response rates by 25 fold for more than five questions (OR: 25, p=0.0001) and increased the correct response rates by 10 fold for more than seven questions (OR: 10, p=0.0001).

Conclusion: In this study, video-based learning was found more successful than the text-based learning as a learning method.

Keywords: Video-based learning, text-based learning, success, medical education, correct response rate

ÖZ

Amaç: Bu çalışma, tıp fakültesi öğrencileri arasında elektronik eğitimin öğrencilerin öğrenme düzeyleri üzerindeki etkisini değerlendirmek ve video tabanlı elektronik öğrenmeyi metin tabanlı öğrenme ile karşılaştırmak için tasarlanmıştır.

Gereç ve Yöntem: Bu çalışma tek merkezli, kesitsel bir çalışma olarak yapılmıştır. Gönüllülerin tamamı tıp fakültesinin dördüncü sınıf öğrencileridir ve tüm öğrenciler rastgele iki gruba ayrıldı. Her gruba 100 öğrenci dahil edildi. Grup 1'de tüm öğrencilere yara iyileşmesi hakkında video tabanlı elektronik öğrenme ile eğitim verildi ve 2. gruptaki tüm öğrenciler ders kitabından standart derslerle eğitildi. Her iki grup da doğru yanıt oranlarına göre değerlendirildi.

Bulgular: Video tabanlı öğrenme, beşten fazla soru için doğru yanıt oranlarını 25 kat arttırırken (OR: 25, p=0.0001) yediden fazla soru için 10 kat arttırdığı tespit edilmiştir (OR: 10, p=0.0001).

Sonuç: Bu çalışmada video temelli öğrenme, bir öğrenme yöntemi olarak metin temelli öğrenmeye göre daha başarılı bulunmuştur.

Anahtar Kelimeler: Video temelli öğrenme, metin temelli öğrenme, başarı, tıp eğitimi, doğru yanıt oranı

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INTRODUCTION

There are 2 main factors in students' motivation to learn, which are described by Sobral (1) as intrinsic and extrinsic factors. Intrinsic factors, in other words autonomous factors, are basically the student's learning motivation and learning styles while extrinsic factors are controllable factors, with teaching methods being one of these factors.

Learning styles have been a common concept in the literature for more than 30 years, and there are different definitions (2). Learning styles are categorized in different ways that address how individuals learn. Barbe and his colleagues classified them into 3 main categories: visual, auditory, and kinesthetic learning (3). Keefe et al. (4) described learning styles as combinations of physiological, cognitive, and emotional characteristics that influence a student's learning. This perspective influences how a learner perceives the learning environment, how they respond to it, and how they react to it.

Dunn and Dunn have developed different teaching methods according to their learning styles. They redesigned their classes, started small group trainings, and used different teaching methods (5). In the perception of the lecturer's emotional state, both visual and auditory cues play a role. The video-based learning contains both visual and auditory stimuli and appeals to both visual and auditory learning.

The purpose of this study was to assess the impact of electronic education on students' learning levels and to compare the video-based electronic learning with text-based learning among medical faculty students.

MATERIAL AND METHODS

Study Design

In this single-center, cross-sectional study, there were 200 fourth-year medical students of Istanbul University, Istanbul Faculty of Medicine. All students were randomly divided into two groups with 100 students in each. Group 1 (n = 100) consisted of students who got video-assisted lectures, and Group 2 (n=100) consisted of students who got lectures from a textbook.

All students had their training about wound healing. The content of the theoretical training for the two groups was exactly the same. While the training was provided to the first group of students with video assistance, the second group was given this training as text reading. This study was blind for all of the students. All medical students only knew the scope, lessons, and exams and believed that this whole process was a part of a routine. Additionally, each group did not know that they were being compared with the other exam-lesson group. 7 days after the video-assisted and text-based lectures, all participants were examined. Groups were evaluated according to the correct response rates. Informed consent was taken from all students. Ethical approval for this study was given by the Istanbul School of Medicine Ethical Committee at Istanbul University.

Statistical Analysis

Continuous variables were analyzed in terms of normal distribution. Normally distributed variables were examined using Student's t-test, and values with non-normal distribution were examined using the non-parametric Mann-Whitney U test. The proportions were compared using $\chi 2$ or Fisher's exact tests. Pearson's $\chi 2$ was performed for dichotomous variables, and Student's t-test for continuous variables.

Binary logistic regression models were used to compare the impact of effectiveness of video-based learning and text-based learning. The significant variables which were detected after $\chi 2$ exact test were included in the binary models.

RESULTS

There were 200 students included in this study, 100 in each group. The mean age of students in group 1 was 24±4 years (20-28) and 23±3 years (20-26) in group 2. The female/male ratio were the same (3:2) for groups 1 and 2, respectively. There was no significantly detected difference between the groups in terms of gender and age (p>0.05) (Table 1).

The mean correct response rate among the students was analyzed, and in group 1, the correct response rate was significantly higher than group 2 (7.9±1.2, 5.3±1.4, respectively) (p<0.001) (Table 1). Therefore, the correct response rate was significant-

Table 1. Demographic features and correct response rates of both groups.

	Video-based learning group n=100	Text-based learning group n=100	p
Age (years) mean±SD (min-max)	24±4 (20-28)	23±3 (20-26)	>0.05
Female/Male	65/35	70/30	>0.05
Correct response rate mean±SD (min-max)	7.9±1.2 (5-10)	5.3±1.4 (3-9)	**<0.001**
Correct response rate for >5 questions (%)	97	41	**<0.001**
Correct response rate for >7 questions (%)	60	7	**<0.001**

ly and positively correlated with video-based learning group (r=0.661, p=0.001).

The evaluation of correct response rates with more than five and seven questions;

In the analysis of correct response rates with more than five questions, we have found that the students in group 1 had significantly higher rates than that of group 2 (97%, 41%, respectively) (p<0.001). The correct response rates with more than seven questions in group 1 was also associated with significantly higher rates than in group 2 (60%, 7%, respectively) (p<0.001) (Table 1).

According to logistic regression analysis, the video-based learning increased the correct response rates 25-fold with five questions (OR:25, p=0.0001) and increased the correct response rates 10-fold with seven questions (OR:10, p=0.0001).

DISCUSSION

This study was performed to compare the effectivity of video-based and text-based learning methods. This comparison was particularly done for the similar homogeneous groups simultaneously in different classrooms. Videos are useful for showing spoken language and moving images, plus many effects like superimposed text, slow motion clips, or animations. All audiences can observe optimally by visualizations remains visible (6). Moreover, video can potentially overcome the shortcomings that simple printed illustrations have. Also, presenting videos to students before starting the course helps to encourage a kind of "blended learning" (7).

When teaching methods are adapted to audiences' learning styles, motivation and performance increase (8-12). Similarly in our study, we found that correct response rates with more than five questions was significantly higher in group 1 (97%) than in group 2 (41%) (p<0.001). Additionally, we found that the rate with more than seven questions was also significantly higher in group 1 (60%) than in group 2 (7%) (p<0.001). We think that, particularly for surgical education, video-based learning is a crucial factor for students' motivations. According to publications in the literature, active learning strategies end with better learning, and active learning reaches all kinds of students (13-15). Therefore, active learning strategies cause reasoning while thinking, develop problem-solving abilities, and can also be used in large classrooms with activities like collaborative learning exercises, simulations, role play, games, and discussion (13). These activities increase motivation and encourage group working.

Recent neuroscience studies have also shown that meaningful developments in learning can be achieved if the learning environments conform to dominant learning styles, a concept known as the "network hypothesis" (16). The most common unimodal selection is kinesthetic, after which visual, auditory, reading, and writing are listed (17). Students who learn kinetics prefer hands-on learning and enjoy learning that has a connection with reality (18,19). Kinesthetic learners should pay more attention to experience to be better informed. They more frequently select simulations of practical applications, examples, exhibitions, photographs, "real-life examples", role play, and applications that facilitate their understanding of principles and advanced concepts (20).

According to research conducted by Kharb et al. at a medical university in India, 61% of the students preferred the multimodal VARK (Visual, auditory, read/write and kinesthetic). 39% of the participants preferred unimodal learning, 41% bimodal, 14% trimodal, and 6% quadrimodal learning (18). We believe that the bimodal learning sytem, video-based learning style, is more successful than other unimodal learning styles. In another study, Slater et al. (21) reported that Wayne State University's first-year medical students, consisting of both male and female participants, preferred to utilize multiple sensory modalities over unimodal learning. Although visual preference was common for both genders, it was not statistically significant. We also didn't find any significance between both genders (p>0.05). Age has been accepted as a factor in the learning method shifting from one to another (22). However, as age increased, there were significant differences in visual and reading/writing learning style preferences. In this study, it was found that as the subjects got older, their preferences for kinesthetic and auditory modalities increased and their visual and reading/writing presentation preferences decreased in the same model (10,23).

Although it is not known exactly whether the difference in learning styles is due to different levels of medical education or to increasing age, it required further research by taking both factors into account (24).

However, in this study, age was not found to be significant between both groups.

We attribute this to the close distribution of the age between the 2 groups.

In a study conducted by Samarakoon and et al.(25), while first year medical students preferred auditory and reading/writing learning strategies, multimodal learning styles were preferred by senior students. According to logistic regression analysis, video-based learning increased the correct response rates 25-fold with more than five questions (OR: 25, p=0.0001) and 10-fold with more than seven questions (OR: 10, p=0.0001).

While the video includes both visual and auditory perceptions, the text-reading just includes visual perception. Moreover, in terms of the text-reading, this visual perception cannot include any motion or depth perception.

CONCLUSION

Visual perception is crucial in surgical education. Additionally, auditory perception is another important cornerstone. As a result, training with videos may make a significant remarkable difference in learning surgical tissue plans. In the comparison of learning modalities between video-based (bimodal modal-

ity) and text-reading (unimodal modality) lectures in medical faculty students, our study showed that video-based learning was more successful than the text-based learning as a teaching method in medical faculty training.

Ethics Committee Approval: Ethical approval for this study was given by the Istanbul School of Medicine Ethics Committee at Istanbul University.

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DERLEME
REVIEW

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Endojen Opioidlerin Ağrı Mekanizması Üzerine Etkileri

Effects of Endogenous Opioids on Pain Mechanism

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ÖZ

Ağrı, "gerçek veya potansiyel doku hasarı ile ilişkili tarif edilen hoş olmayan bir duyusal ve duygusal deneyimdir" şeklinde tanımlanmaktadır. Opioidlerin ağrı mekanizması üzerine etkilerini ortaya koymak amacıyla birçok çalışma yapılmıştır. Bu çalışmaların çoğunda opioid peptidleri ve opioid reseptör agonistleri üzerine odaklanılmıştır. Ağrı ile dopaminerjik, serotonerjik, glutaminerjik sistemlerin yanı sıra opioid sisteminin de ilişkili olduğu belirtilmektedir.

Papaver somniferum bitkisinden elde edilen ve bir opioid çeşidi olan morfin uzun yıllardır ağrı tedavisinde kullanılmaktadır. Morfin ve diğer ekzojen opioidler canlı organizmada opioid reseptörlerine bağlanarak aktivite gösterirler. Bu reseptörler, proteolitik parçalanma yoluyla aktive olan inaktif polipeptit hormonları olarak ifade edilen opioid peptitlere yanıt verir. Ekzojen opioidlerin yanı sıra canlı organizmalarda bulunan bu opioidlere endojen opioidler denir. Endojen opioidler ve onların peptidleri, periferik ve merkezi sinir sistemi boyunca eksprese edilir ve birçok farklı sistem ve işlevi düzenler. Endojen opioid peptidlerin temel işlevlerinden biri, ağrıya verilen yanıtı, azalan ağrı yolağı ile modüle etmektir. Bu bilgilerin ışığında endojen opioid mekanizması üzerinden hareketle ağrı mekanizması aydınlatılmaya çalışılmıştır. Bu derlemede ağrı tanımı yapıldıktan sonra endojen opioidler ve reseptörlerinden bahsedilecek ve ağrı araştırmalarında uygulanan metodoloji ve güncel bulgular anlatılacaktır.

Anahtar Kelimeler: Ağrı, opioidler, opioid reseptörleri

ABSTRACT

Pain is defined as an unpleasant sensory and emotional experience, associated with potential tissue damage. Many studies have been conducted to reveal the effects of opioids on the pain mechanism. These studies have focused primarily on opioid peptides and opioid receptor agonists. In general, it is declared that dopaminergic, serotonergic, and glutaminergic systems as well as the opioid system are closely associated with pain.

Morphine, an opioid type obtained from *Papaver somniferum*, has been used for pain relief for many years. Morphine and other exogenous opioids show their activity by binding to opioid receptors in living organisms. These receptors respond to opioid peptides expressed as inactive polypeptide hormones activated by proteolytic cleavage. These opioids found in living organisms are referred as endogenous opioids. Endogenous opioids and their peptides are expressed throughout the peripheral and central nervous system and regulate many different systems and functions. One of the main functions of the endogenous opioid peptide is to modulate our pain response through a descending pain modifying pathway. In light of this data, the mechanism of pain has been elucidated by studying the endogenous opioid mechanism. In this review, after the definition of pain, endogenous opioids and their receptors will be mentioned, the methodology and recent findings in pain research will be explained.

Keywords: Pain, opioids, opioid receptors

Ağrının Tanımı

Uluslararası Ağrı Araştırmaları Derneği (IASP) tarafından ağrı, "gerçek veya potansiyel doku hasarı ile ilişkili veya bu tür hasarlar açısından tarif edilen hoş olmayan bir duyusal ve duygusal deneyimdir" şeklinde tanımlanmaktadır. Ağrı, fizyolojisine, yoğunluğuna, süresine, etkilenen doku türü ve sendromlarına göre sınıflandırılabilir. Fizyolojisine ya da mekanizmasına göre ağrı, nosiseptif, nöropatik ve enflamatuvar; yoğunluğuna göre hafif-orta-şiddetli; seyrine göre akut, kronik; ilgili doku tipine göre cilt, kaslar, iç organlar, eklemler, tendonlar, kemikler; sendromlarına göre kanser, fibromiyalji, migren ağrısı şeklinde sınıflandırılır. Bunların yanı sıra psikolojik durum, yaş, cinsiyet ve kültür gibi özel hususlar da ağrı oluşumunda etkilidir (1).

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Ağrının Fonksiyonu

Ağrının temel işlevi organizmayı zarardan koruyan ya da yaralanmayı en aza indirmeyi sağlayan bir uyarı sinyali olmaktır. Canlı organizmalarda ağrı reseptörleri (nosiseptörler) yaygındır. Nosiseptör ağı, derinin herhangi bir yerinden, kaslar ve iç organlar dahil daha derin dokulardaki hasarın sinyalini verebilir. Nosiseptörler, soğuk, ısı, basınç ve kimyasal uyarım gibi bir dizi modaliteye duyarlılığı olan sistemlerden meydana gelir. Ağrının ikinci ana işlevi yaralanmadan sonra ortaya çıkar. Organizmadaki içsel mekanizmalar ağrı şiddetini, yerini ve süresini etkiler. Ağrı, yaralı bölgeden yaralanmamış bölgelere yayılır ve iyileşme sırasında devam eder (1).

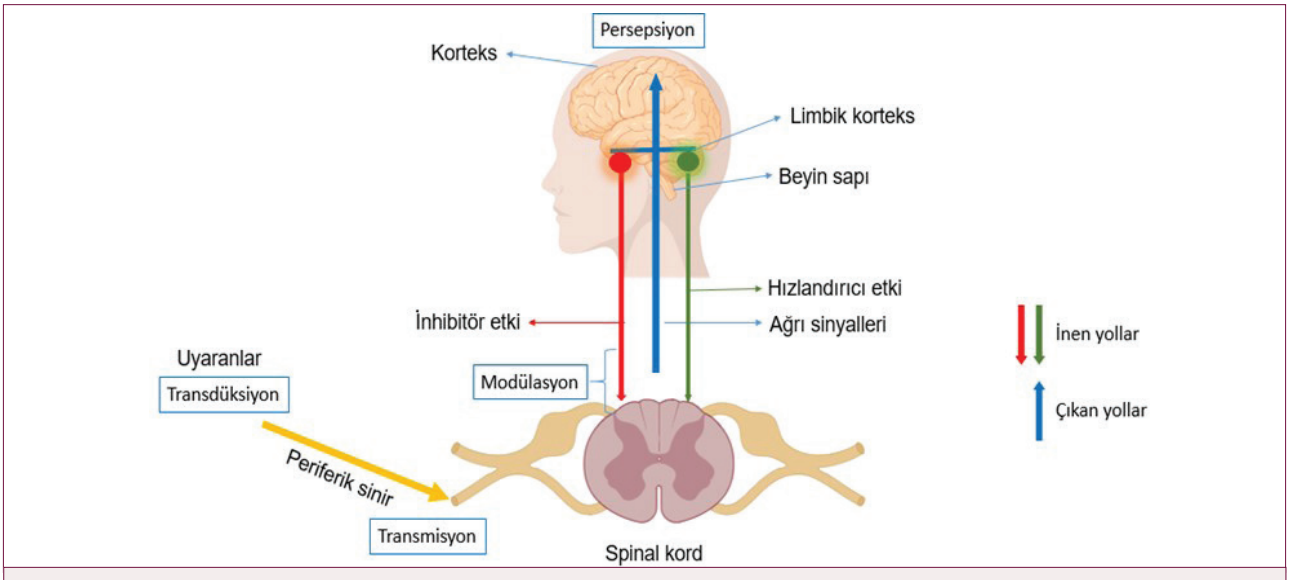
Ağrı mekanizması

Ağrı ilk olarak periferik nosiseptörlerin aktivasyonuyla başlar. Bu reseptörler, Aδ ve C duyusal aferent sinirleri uyaran bir dizi aljezik ve hiperaljezik aracılar tarafından aktive edilir. Duyusal bilgi, β-endorfin ve metenkefalin tarafından modüle edildiği omuriliğin çevresinden dorsal boynuzuna iletilir ve bu, savunma davranışında önemli bir rol oynar (2). Ağrının iletimi transdüksiyon, transmisyon, modülasyon ve persepsiyon olmak üzere dört aşamada gerçekleşir. *Transdüksiyon;* nosiseptörlerde ağrılı uyaranın elektriksel aktiviteye dönüştürüldüğü aşamadır. *Transmisyon;* nosiseptif impulsun sinir sistemi boyunca iletilmesidir. Transmisyon esnasında, öncelikle primer sensöriyel afferent nöronlar elektriksel aktiviteyi omuriliğe taşır. Bunu takiben, nosiseptif impulslar medulla spinalisten çıkan ileti sistemi vasıtasıyla beyin sapı ve talamusa ulaştırılır. Son olarak, talamustan talamokortikal bağlantılarla somatosensöriyel kortekse iletilir. *Modülasyon;* nosiseptif transmisyonun nöral etkenlerle modifiye olmasıdır. *Persepsiyon* ise bireyin psikolojisi ile etkileşimi ve subjektif emosyonel deneyimleri sonucu gelişen, uyarının algılandığı son aşamadır (Şekil 1) (3).

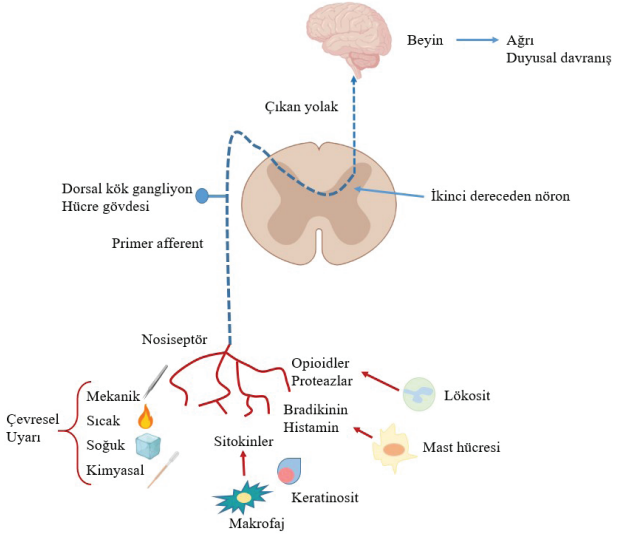
Cilt, kas, vissera, kemik gibi birçok yerde bulunan nosiseptörlerin devreye girmesi ile uyarılan nöronlardan glutamat gibi eksitatör nörotransmitterlerin salınması ağrıyı arttırıcı, gama aminobütirik asit (GABA) gibi inhibitör nörotransmitterlerin salınması ise ağrıyı azaltıcı yönde etki yapar (4). Spinal kord, periferden gelen ağrı sinyallerini çıkan yollar (afferent) vasıtasıyla üst merkezlere iletir. Kortekse ulaşan bu sinyaller, üst merkezlerde değerlendirildikten sonra beyin sapı yoluyla spinal korda geri dönerek ağrıyı kontrol eden inen yolları (efferent) oluşturur. İnen yollar, noradrenalin, seratonin ve dopamin gibi monoaminleri kullandığı için monoaminerjik bir yolaktır. Bu yollar ağrıyı azaltan özellikte olmasının yanı sıra ağrıyı reseptör alt tipi ve baskın monoamin tipine göre arttırabilmektedirler. Bu yüzden, bu yolak modülasyon yolağı olarak da adlandırılır (5).

Ağrı, nosiseptör adı verilen spesifik reseptörler tarafından algılanır. Nosiseptörler periferik terminalleri ağrılı uyaranlara hassas, deri ile deri altı dokularında bulunan çıplak ve serbest sinir uçlarıdır. Nosiseptörler tarafından tespit edilen zararlı sinyaller işlenmek üzere beyine iletilir (6-9). Nosisepsiyon, bir hayvanı veya insanı olası hasara karşı uyaran temel duyu sistemidir. Nosiseptif yollar, motivasyon için önemli olan beyin bölgeleri ile bağlantılıdır ve hayvanlar, kendilerini zararlı uyarandan ve hasardan korumak için sinir sistemi tarafından uyarılır. Evrimsel sürece ve türlerin yaşam şartlarına bağlı olarak türler farklı nosiseptif uyarılara (yüksek mekanik basınç, aşırı sıcaklıklar, zararlı kimyasallar) maruz kalırlar. Bu nedenle türler de çevrelerine uyum sağlamak için nosiseptif sistemlerini geliştirirler (10).

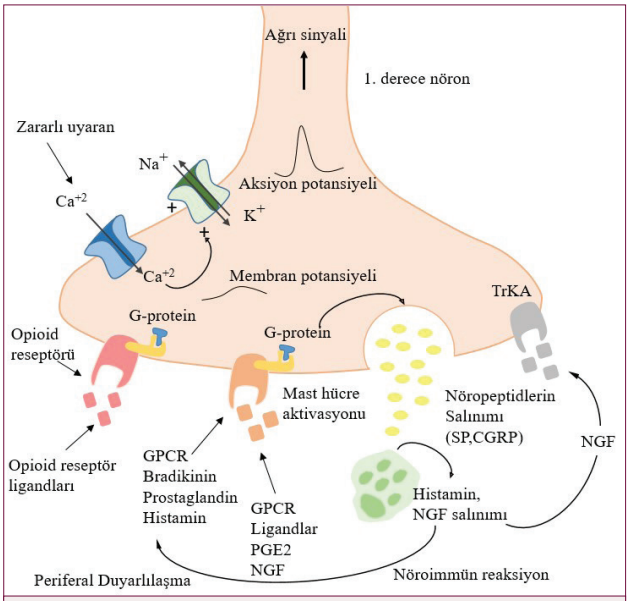
Ağrıya duyarlı nosiseptörlerin aktivasyonu sonucu nörokimyasallar (endorfin, serotonin, P maddesi, bradikinin) salgılanır (Şekil 2-3) (11,12). Nosiseptörler ile bunları çevreleyen düz kaslar, kapiller ve aferent sempatik sinir uçları nosiseptörlerin mikro çevresini oluşturur. Nosiseptörler mekanik uyaranlarla uyarılmalarına ek olarak, endojen algojenik madde olarak tanımla-



Şekil 1. Ağrının algılanma mekanizması (3).



Şekil 2. Periferden beyne giden ağrı yolu: Zararlı çevresel uyaranlar, ağrı hissine yol açan periferik nosiseptörleri aktive edebilir. Sinir terminalinin yakınında bulunan mast hücresi, lökositler, makrofajlar, keratinositler gibi çeşitli hücreler, sinirin aktivasyon eşiğini yükselten veya azaltan aracıları serbest bırakabilir (11).



Şekil 3. Periferde nosiseptör duyarlılığının özellikleri: Termal, kimyasal veya mekanik kaynaklı zararlı bir uyarı, merkezi sinir sistemine giden aksiyon potansiyellerini ortaya çıkarmak için kalsiyum ve sodyum kanallarıyla güçlendirilmiş bir elektrik sinyaline dönüştürülür. Bir yaralanmadan sonra sinir terminaline yakın mast hücreleri aktive olur. Bu, histamin, sinir büyüme faktörü (NGF), prostaglandin ve bradikinin gibi enflamatuar aracıların salınmasına yol açar. Bunlar, GPCR ve tirozin kinaz reseptörü tip 1 (TrKA) gibi, nosiseptor sinir terminallerinde ifade edilen reseptörler üzerinde etki ederek periferik nosiseptör duyarlılaşmasına yol açar. Ayrıca, immün hücreler ve nosiseptörler arasında etkileşim ile sonuçlanan nöropeptitlerin (örn: P maddesi (SP) ve kalsitonin gen ilişkili peptid (CGRP)) salımı vardır. PGE2: Prostanglandin E2 (12).

nan P maddesi, bradikinin, histamin, prostaglandinler, lökotrienler, interlökinler ve TNF–α gibi biyokimyasal moleküllerle de düzenlenebilirler (13).

Endojen Opioidler

Endojen opioidler, G proteinine bağlı reseptör (GPCR) süper ailesine ait olan spesifik opioid reseptörleri üzerinde etki ederler (14,15). Endojen opioid sistemi klasik μ opioid peptid (MOP), δ opioid peptid (DOP) ve κ opioid peptid (KOP) reseptörleri ve klasik olmayan Nosiseptin/Orfanin FQ/NOP reseptöründen oluşur. Bunun yanı sıra, opioid peptidleri olan enkefalin, β-endorfin, dinorfin ve endomorfinden oluşur. MOP, DOP ve KOP opioid reseptörleri sırasıyla OPRM1, OPRK1 ve OPRD1 genleri tarafından kodlanır (Tablo 1) (16). FQ/NOP reseptörlerinin mekanizması henüz tam olarak aydınlatılmamıştır. Klasik opioid reseptörleri naloksonun antagonistik etkisine ve endojen opioidlerin agonistlerine duyarlıyken, klasik olmayan opioid reseptörleri duyarlı değildir (17). Opioid peptidleri, sırasıyla spesifik öncüleri olan preproenkefalin (PENK), proopiomelanokortin

Tablo 1: Endojen opioid sistemin karakterizasyonu (16).

	Endojen Agonistler		
Opioid reseptör	**Öncül**	**Peptit**	**Ağrıya Etkisi**
MOP (μ)	POMC	Endorfin* Endomorfin-1 Endomorfin-2	Analjezi
DOP (δ)	PENK	Enkefalinler*	Analjezi
KOP (κ)	PDYN	Dinorfinler	Analjezi
FQ/NOP	ppN/OFQ	Nosiseptin/orfanin FQ	Analjezi, hiperaljezi/anti-opioid cevabı (beyinde)

*Endorfinler ayrıca DOP reseptörlerine de bağlanırken; enkefalinler MOP reseptörlerine bağlanır.

(POMC), preprodinorfinden (PDYN) meydana gelirler (18). Enkefalinler ve β-endorfin, MOP ve DOP reseptörlerinin endojen ligandlarıdır. β-endorfin, MOP ve DOP reseptörlerini yaklaşık olarak eşit afinite ile bağlarken, enkefalin, DOP reseptörü için on kattan daha fazla yüksek afiniteye sahiptir. Dinorfinlerin seçici olarak KOP reseptörlerini ve bunun yanında birkaç opioid olmayan reseptörü de aktive ettiği belirtilmektedir (19).

Endojen Opioid Peptidleri: β-endorfin, Enkefalin ve Dinorfin, Orfanin FQ/Nosiseptin

Opioid peptidler temel olarak PENK, PDYN, POMC ve pronosiseptin (PNOC) olmak üzere dört öncüden türetilir (Şekil 4) (20). En yaygın olarak bulunan opioid peptidler, POMC adı verilen tek bir prohormondan türetilen endorfinler iken (en güçlü form, β-Endorfin), metiyonin-enkefalin (M-ENK; Tyr-Gly-Gly-Phe-Met) ve lösin-enkefalin (L-ENK; Tyr-Gly-Gly-Phe-Leu) formunda bulunan enkefalinler, M-ENK'nın C-terminali PENK ve PDYN' den türetilir (21). PDYN, dinorfin A (DYN-A), dinorfin B (DYN-B) ve neoendorfin enkefalinlere karşılık gelen iki motiften oluşur. Endomorfinlerin ise öncü proteinleri henüz tanımlanmamıştır (22).

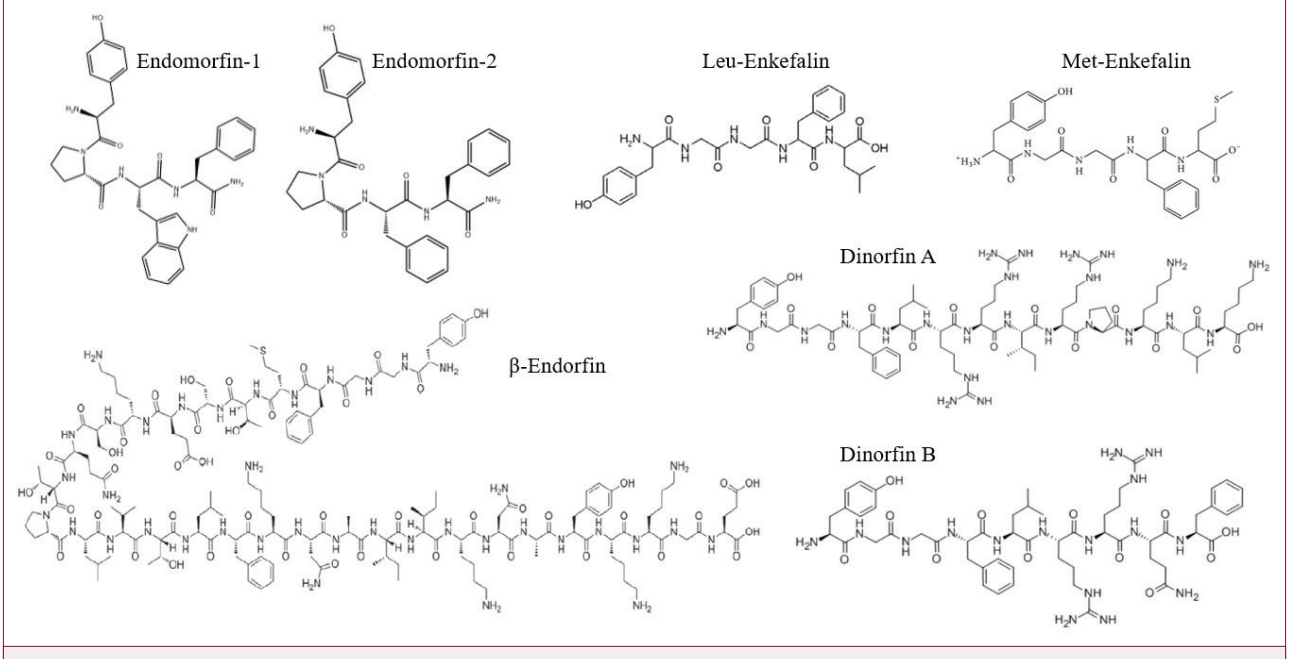
Hughes ve ark. (1975) beyinde μ reseptörlerini bağlayabilen iki pentapeptid (met- ve leu-enkefalinler) olduğunu ortaya koymuşlardır. Bu gözlemleri β-endorfin ve dinorfinlerin keşfi izlemiş ve bunların canlılardaki ağrı giderici sistemde ve bağışıklık sinyalizasyonundaki rollerinin gösterilmesi takip etmiştir (23,24). Özellikle bazal gangliyonlarda yaygın olarak bulunan β-endorfin, enkefalin ve dinorfin gibi opioid peptidler nöromodülatör olarak fonksiyon gösterirler. Bu peptitler işlevlerini μ, δ ve κ opioid reseptör alt tipleri üzerindeki etkileri aracılığıyla gerçekleştirirler (25).

Enkefalinler bağırsak, sempatik sinir sistemi ve adrenal bezlerde üretilir ve DOP reseptörleri için başlıca endojen agonistlerdir (26). Enkefalinler, ilk olarak memelilerde keşfedilen pentapeptidlerdir (23). PENK geninin translasyon sonrası işlenmesi, altı Met-ENK kopyası ve bir Leu-ENK kopyası içeren yedi opioid dizisini kodlar (20). Bununla birlikte, Met-ENK kopyalarının sayısı değişkendir ve Leu-ENK, daha düşük omurgalı taksonlarında her zaman PENK geninin bir parçası olmamaktadır.

β endorfin, 31 amino asit içeren ve μ reseptörüne etki eden birincil endojen opioiddir (27). β-endorfin, öncü peptit POMC' den üretilen bir peptittir. POMC, sadece ödül, doygunluk ve ağrıya değil aynı zamanda hipofiz ve adrenal ekseni düzenleyen hormonal sistemlere de aracılık eden sistemler açısından zengin bir alan olan hipotalamusta da üretilmektedir (26,28).

κ opioid reseptörü için endojen peptit olan dinorfin, birçok forma sahip karmaşık bir nöropeptiddir. Bu nöropeptid, öncelikle ödül arama, motivasyon işleme, stres tepkisi ve ağrı duyarlılığını düzenleyen ve böylece madde ve alkol kullanım bozukluğunun gelişimini etkileyen nöronal yolakları modüle eder (29). Dinorfin beyinde birçok bölgede yoğun olarak bulunmasının yanı sıra amigdala, entorinal korteks, dentat girus, hipotalamus, orta beyin, striatum, hipokampus ve medulla-ponsta (29) serebellum ve kortekse oranla daha yüksek seviyelerde bulunur (29).

Kronik ağrı, amigdaladaki κ opioid reseptörü üzerinde stresi uyaran endojen dinorfin aracılı etkilerle ilişkilidir. κ opioid reseptörünün blokajının, ameliyatla ilişkili anksiyetenin ve ameliyat öncesi artan stresin sonuçlarının önlenmesinde de faydalı olabileceği düşünülmektedir (30).



Şekil 4. Endojen opioid peptidlerinin moleküler yapıları.

Endomorfinler, MOR için afinitesi olan bir opioid peptidler sınıfına aittir ve ilk olarak sığır beyninde ve insan korteksinde keşfedilmiştir (31). İki endomorfin formu tanımlanmıştır; endomorfin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) ve endomorfin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2). EM-1 ve EM-2 amino asit dizilerindeki diğer opioid peptidlerden farklılıkları olmasına rağmen, kodlayan haberci molekülü bilinmemektedir. Güçlü bir antinosiseptif etkiye sahip olmanın yanı sıra, endomorfinlerin memelilerde otonomik, nöroendokrin ve ödül işlevlerinin modülasyonunda rol oynadığı belirtilmiştir (32).

On yedi amino asit içeren bir peptit olan Orfanin FQ/nosiseptin (OFQ/N), opioidlerle yapısal olarak büyük oranda benzerlik göstermesinin yanı sıra yapılan bir dizi çalışmada farmakolojik olarak opioidlerden farklı olduğu belirtilmektedir. Aynı zamanda yine literatüre bakıldığında hem merkezi hem de periferik sinir sisteminde ve ayrıca nöronal olmayan dokularda opioid sistemin temel fonksiyonlarından olan nosisepsiyon modülasyonu (33-35), lokomotor aktivitede etkinlik, nörotransmitter ve hormon salınımının regülasyonu, hafıza ve öğrenme modülasyonu (35) gibi çeşitli fizyolojik fonksiyonlara da sahip olduğu belirlenmiştir. Tüm bunların ışığında OFQ/N opioid sistem ile farmakolojik olarak farklılık göstermesine rağmen yapısal ve fizyolojik fonksiyon bakımından benzerliklerinin olduğu da görülmektedir.

Opioid Reseptörler

MOP, DOP, KOP ve NOP reseptörlerinin aktivasyonu, adenilat siklazların ve siklik adenozin monofosfat (cAMP) oluşumunun G proteinlerine bağımlı inhibisyonuna neden olabilir (17). Bununla birlikte, bu etkilerin ağrı inhibisyonu ile sonuçlandığı kesin yol, sadece MOP reseptörleri için açıklanmıştır. Bu nedenle, azalmış cAMP üretimi, protein kinaz A (PKA) aktivitesinin inhibisyonuna yol açar, bu da ağrının azalmasında rol oynayan çeşitli iyon kanallarının baskılanmasıyla sonuçlanır. Bu kanallar arasında ısı sensörü geçici reseptör potansiyel katyon kanalı alt ailesi V üyesi 1 (TRPV1), hiperpolarizasyonla aktive edilmiş siklik nükleotid-kapılı (HCN) kanallar, asit algılayıcı iyon kanalları (ASIC) ve voltaj kapılı Na^+ (Nav) kanalları yer alır (Şekil 5)(17,36).

***Mü (μ) opioid reseptörü** (MOR)*

Morfin için primer bağlanma bölgesi olduğunun bulunmasının ardından bu ismi almıştır. MOR, opioidlerin antinosiseptif özelliklerinin yanı sıra, kannabinoidler, alkol ve nikotin gibi opioid olmayan uyarıcı/uyuşturucu ilaçların ödüllendirici yönlerinin yanı sıra sosyal etkileşim gibi faaliyetlerine de aracılık eder (26). Bu reseptörler, supraspinal lokasyonlarda, özellikle presinaptik bölgelerde yüksek yoğunlukta bulunur ve genellikle çıkan yolları etkisiz hale getirirken inen yolları etkinleştirir. Limbik sistemde ve hipotalamus gibi nörohormonal sekresyonla ilişkili beyin bölgelerinde yüksek konsantrasyonda bulunurlar. Yukarıda belirtilen işlevlere ek olarak, μ reseptörleri ağrı ve strese yanıtın düzenlenmesinde önemli işlevlere sahiptir (26). μ reseptörü, ağrıyı tedavi etmek için kullanılan güncel opioid agonistlerinin çoğunun birincil hedefidir.

***Kappa (κ) opioid reseptörü** (KOP)*

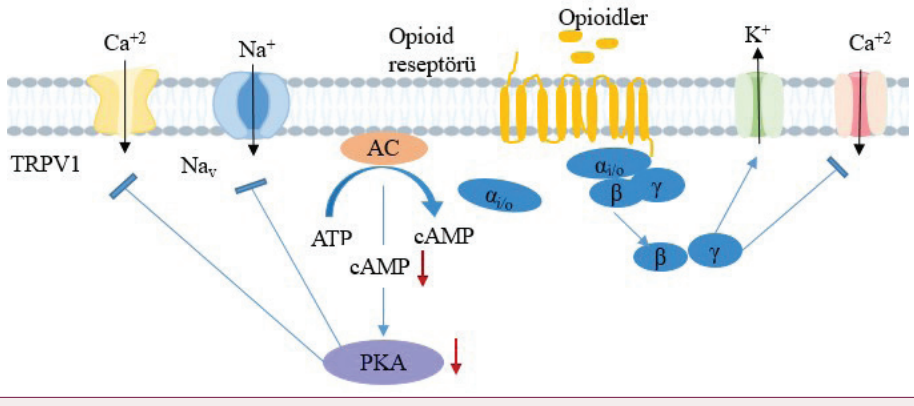
KOR'ların, μ reseptörü ile karşılaştırıldığında farklı fizyolojik süreçlere aracılık ettiği düşünülmektedir. Omurilikte daha yüksek yoğunlukta bulunurlar. Hiperaljezi üretiminde önemli rol oynamalarının yanı sıra viseral ağrının modülasyonunda da etkili oldukları düşünülmektedir. Bununla birlikte, bugüne kadar çalışılan κ agonistlerinin, ilaç gelişimini sınırlayan bir özellik olan güçlü disforik özelliğe sahip oldukları da belirtilmektedir (37).

***Delta (δ) opioid Reseptörü** (DOP)*

Üçüncü tip opioid reseptörü, δ reseptörüdür. μ reseptörleri ile kıyaslandığında, δ opioid reseptörleri orta beyinde nispeten düşük konsantrasyonlarda bulunurken, periferik sinirlerin dorsal kök ganglionlarında yüksek yoğunlukta bulunur. Akut ağrı algısı üzerinde daha düşük etkiye sahiptirler. Ancak periferdeki kronik ağrı ve nosisepsiyonu modüle etmede daha önemli bir role sahip olabilecekleri düşünüldüğü için güncel farmakolojik araştırmaların önemli bir hedefi olarak görülmektedirler (14,26,38).

Nosiseptin/Orfanin FQ (NOP) Reseptörü

Klasik olmayan NOP reseptörleri, nalokson ile antagonizmaya karşı duyarsızdır. Opioid peptidlere karşı düşük afiniteye sahip olup seçici endojen agonistleri, prepro-N/OFQ'dan türeyen no-



Şekil 5. Opioid sinyal yolağı (36).

siseptin/orfanindir. Beyindeki NOP reseptörlerinin aktivasyonu, hayvan modellerinde hiperaljezi veya anti-opioid etkilere yol açabilmektedir (33,34,39).

Klinik ve Deneysel Ağrı Araştırmaları

Opioidlerin ağrı mekanizması üzerine etkilerini ortaya koymak amacıyla birçok çalışma yapılmıştır. Bu çalışmaların birçoğunda opioid reseptör agonistleri üzerine odaklanılmıştır. Furst ve ark. yaptıkları çalışmada, enflamasyon kaynaklı ağrının hayvan modellerinde periferal MOR, DOR ve KOR'un baskılanmasına katkı sağladığını belirtmişlerdir. Yapılan çalışmada, sıçanlarda formalin testinin enflamatuvar aşamasında, subkutan olarak uygulanan yeni periferik olarak kısıtlanmış μ-opioid agonistlerin, doza bağlı antinosisepsiyon geliştirdiği görülmüştür. Bu etkinin periferal MOR'un aktivasyonundan kaynaklandığını göstermek için de periferik olarak kısıtlanmış antagonist nalokson metiyodid kullanılmış ve tersine etki görülerek MOR'un etkisi teyit edilmiştir (40). Pacheco ve Duarte yaptıkları çalışmada, DOR agonisti SNC80'i, subkutan uygulamayı takiben sıçan pençesinin PGE2 ile indüklenen hiperaljezisinde doza bağlı antinosisepsiyon ürettiğini görmüşlerdir (41). Bu etki, aynı uygulamanın pençeye uzak bir bölgeye uygulanmasından sonra da gözlenmiş, ve elde edilen sonuç, antinosisepsiyonun periferal DOR tarafından sağlandığını düşündürmüştür (41). Binder ve ark. yaptıkları çalışmada, KOR seçici opioid agonistleri pençeye deri altından uygulandığında, Freund'un adjuvanla (FCA) indüklenen tam kronik enflamasyondan sonra ağrı eşiklerinde, nalokson metiyodid tarafından antagonize edilen güçlü doza bağlı artışlara neden olduğunu görmüşlerdir (42). Sengupta ve ark. enflamatuvar bağırsak hastalığı oluşturdukları bir hayvan modelinde, periferik olarak kısıtlanmış κopioid agonistler, viskeromotor tepkilerde ve afferent sinir aktivitesinde doza bağlı, çevresel olarak antagonize azalmalar oluşturarak periferik KOR'un antinosisepsiyonu etkilediğine dair kanıtlar ortaya koymuşlardır (43). Sıçan pençesinin FCA ile indüklenen enflamasyonunu kullanan tek bir çalışmada, lokal olarak hareket eden MOR, DOR ve KOR seçici agonistlerin, doza bağlı, stereospesifik ve reseptöre özgü antagonistler tarafından geri döndürülebilir olan antinosisepsiyon sağladığı bildirilmiştir (42). Enflamatuvar ağrıya ek olarak, periferal opioid reseptörlerinin termal yaralanmayı takiben sıçanlarda ve uterin servikal distansiyon, nöropatik ağrı ve nosiseptif ağrının hayvan modellerinde nosiseptif tepkileri hafiflettiği bildirilmiştir (40).

Opioidlerin ağrı mekanizması üzerindeki etkilerini ortaya koymak adına opioid peptidleri ile de birçok çalışma yapılmıştır. Yaygın kronik ağrılı HIV pozitif bireylerde β endorfin seviyelerinin azaldığı gösterilmiştir. Bu nedenle HIV pozitif yaygın kronik ağrılı bireylerde tedavi amaçlı olarak endojen opioidleri artırmaya yönelik stratejiler vardır. Pannell ve ark. tarafından bu tür bir strateji kullanılmış, burada daha yüksek seviyelerde endojen opioid içeren makrofajların farelere aktarımı, opioid reseptör antagonisti nalokson metiyodid tarafından tersine çevrilen mekanik aşırı duyarlılığı iyileştirdiği görülmüştür (44).

Benzer şekilde endojen opioidleri artıran peroksizom proliferatör ile aktive olan reseptör (PPAR) agonistlerinin, enflamatuvar ağrı modellerinde nosiseptif eşiği yükselttiği gösterilmiştir (45).

Endojen opioidleri ifade eden nöronları incelemek için çeşitli transgenik modeller geliştirilmiştir. Bu modeller arasında POMC, preproenkefalin, enkefalin ve dinorfin için nakavtlar ve Cre-rekombinaz hatları kullanılmıştır. Bunun yanı sıra, opioid reseptörlerinin rolünü incelemek için MOR, DOR ve KOR nakavt hatlarını içeren farelerde çalışmalar yapılmıştır (45). Opioid peptidlerini ve reseptörlerinin nakavt edilmesi ile davranışsal sonuçlarını tanımlayan ilk çalışmalar, her nakavt hattındaki peptidler ve reseptörlerdeki gelişimsel adaptasyonların tartışılması dahil olmak üzere dikkatle gözden geçirilmiştir. Kısaca, enkefalin nakavt fareler, supraspinal analjezi ve enflamatuvar ağrıda eksikliklere sahiptirler. B-endorfin nakavtları, kronik ağrıdan sonra anormal MOR regülasyonu göstermişler ve dinorfin nakavtları, bozulmuş stres kaynaklı davranış tepkileri sergilemişlerdir (46). Navratilova ve ark. ratlarda yaptıkları çalışmada, kronik ağrının amigdaladaki KOR üzerinde stresi teşvik eden endojen dinorfin aracılı etkileri ile ilişkilendirmiş ve KOR blokajının, ameliyatla ilişkili anksiyeteyi ve ameliyat öncesi artan stresin sonuçlarını önlemede de faydalı olabileceğini ortaya koymuşturlar (30).

Ağrı mekanizması üzerine yapılan farmakolojik çalışmalarda opioidlerin ağrı üzerine etkilerini ortaya koymak adına yapılan çalışmalar da literatürde mevcuttur. Schnörr ve ark. (47) yaptıkları çalışmada, zebra balığı embriyolarına 5 gün boyunca seyreltik asetik asit maruziyeti ve analjezik olarak da buprenorfin uygulayarak zararlı bir uyarana maruz kalmaları üzerine davranışsal tepkiler görmeyi hedeflemişlerdir. Maruziyet sonrası yanıtların büyüklüğü, uygulanan uyaranın yoğunluğuna bağlı olarak nosiseptif yolların aktivasyonu sağlamış ve değişen cox-2 mRNA ekspresyonu ile de doğrulanmıştır. Analjezik buprenorfinin, bu modelde gelişmiş omurgalılarda ve memelilerde olduğu gibi benzer antinosiseptif özelliklere sahip olduğu ve zararlı uyaran tarafından indüklenen davranışsal tepkileri önleyebildiği ortaya konulmuştur. Deakin ve ark. (47) yapmış oldukları çalışmalarda balıklara asetik asit ile ağrı modeli oluşturmuş ve bunu davranış testleri ile de desteklemişlerdir. Bunun yanı sıra balıklara morfin gibi ekzojen opioidler vererek ağrının opioidler aracılığıyla azaltılabildiğini ve yine aynı çalışmalarda balıklara opioid antagonisti olan nalokson vererek morfin etkisinin ortadan kaldırıldığında balıkların yine aynı ya da yakın ağrıları hissettiğini ortaya koyarak balıkların ağrı çalışmaları için uygun bir model olduğunu ortaya koymuşlardır (47).

Farmakolojik olmayan ağrı tedavilerinin analjezik etkilerinin, endojen opioid peptidleri etkilediği belirtilmiştir (48,49). Bu nedenle ağrının giderilmesi amacıyla bir kısmı farmakolojik olmayan tedavi denemeleri bulunmaktadır. Akupunktur ve elektro-akupunktur yüzyıllardır Asya kültüründe tedavi edici bir yöntem olarak kullanılmaktadır. Nöral endojen opioid sistemi, ağrının yönetiminde anahtar bir rol oynar ve akupunktur veya elektro-akupunkturla indüklenen analjezinin aracılığında çok önemlidir. Çalışmalar, düşük frekanslı elektro-akupunkturun

sırasıyla μ-opioid, δ-opioid ve FQ (NOP) reseptörleriyle etkileşime giren β-endorfin, enkefalin ve nosiseptin ekspresyonunu artırdığını ortaya koymuştur. Bununla birlikte, yüksek frekanslı elektro-akupunktur tedavisinin, KOR ile etkileşime giren dinorfin A'nın ekspresyonunu artırdığı bildirilmiştir (49).

SONUÇ

Ağrı üzerine yapılan birçok çalışmada genel olarak ağrı mekanizmasının aydınlatılması amaçlanmıştır. Bu çalışmalarda ağrı ile dopaminerjik, serotonerjik, glutaminerjik sistemlerin yanı sıra opioid sisteminin de ilişkili olduğu belirtilmektedir. Opioidler ekzojen ve endojen olarak ikiye ayrılırlar. Ekzojen bir opioid olan ve analjezik etkiye sahip morfin 19. yüzyılın başlarında keşfedilmiş ve günümüzde de ağrı kesici/analjezik olarak kullanılmaya devam edilmektedir. Morfin en etkili opioid analjeziklerinden biridir; fakat kronik ağrı durumlarında uzun süreli kullanılması sonucu bağımlılık geliştirmesi sebebiyle yan etkiye sahiptir. Bu durum araştırmacıları yeni ve yan etkisi olmayan analjezikler geliştirmeye yönlendirmiştir. Bu amaçla ağrı mekanizmasının bilinmeyen yönlerini aydınlatılması için endojen opioidler üzerine yapılan çalışmalar uzun yıllardır devam etmektedir.

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DERLEME
REVIEW

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Yetişkin Nörogenez ve Nörodejeneratif Hastalıklarda Büyüme Faktörlerinin Rolü

The Role of Growth Factors in Adult Neurogenesis and Neurodegenerative Diseases

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ÖZ

Nörogenez, nöral kök hücrelerin (NKH) bölünme, göç etme ve farklılaşma süreçlerinin bütününü oluşturmaktadır. Lateral ventrikülün subventriküler bölgesi (SVZ) ve hipokampus dentat girus (DG)'daki subgranüler bölge (SGZ) olmak üzere iki farklı nişte gerçekleşmektedir. Nörotrofik faktörler, NKH ve nöral progenitor hücrelerin migrasyon, proliferasyon ve farklılaşmasında rol oynar. Ayrıca, NKH'lerin hasarlı dokuda, nöral hücrelerin yeniden yapılanması, nöral plastisite ve anjiogenezi düzenleyici etkileri olduğu gösterilmiştir. Yetişkin nörogenezinde ise nörotrofik faktör kombinasyonlarının serebrovasküler, nörodejeneratif, onkolojik hastalıklar ve travma sonrası oluşan inflamatuvar hasar tedavisinde önemli rolü olduğu bilinmektedir. Bu derlemede, nörotrofik faktörlerin NKH'ler üzerindeki modüle edici etkisi ve potansiyel terapötik uygulamalarında preklinik ve klinik çalışmaları içeren güncel literatürler biraraya getirilmiştir. Bu alanda çalışma yapan araştırmacı ve hekimlere fayda sağlayacak güncel bilgiler içermektedir.

Anahtar Kelimeler: Nörogenez, nörotrofik faktör, nöral kök hücre

ABSTRACT

Neurogenesis is the combined processes of division, migration and differentiation of neural stem cells (NSCs). The two different locations: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) in the hippocampus dentate gyrus (DG), provide a niche environment for neurogenesis. Neurotrophic factors have roles on migration, proliferation and differentiation of NSC and neural progenitor cells. Studies have shown that NSCs have regulatory effects on neural cell rearrangement, neural plasticity and angiogenesis in damaged tissue. In adult neurogenesis, combinations of neurotrophic factors play an important role in the treatment of cerebrovascular, neurodegenerative, oncological diseases and post-traumatic inflammatory damage. In this review, current literature including pre-clinical and clinical studies for the modulating effect of neurotrophic factors on NSCs and their potential therapeutic treatment applications are brought together. It contains up-to-date information that would be beneficial for researchers and physicians working in this field.

Keywords: Neurogenesis, neurotrophic factor, neural stem cell

GİRİŞ

Kök hücreler, canlı organizmalarda yaşam boyu varlıklarını sürdürerek organizmanın onarım ve yenilenme süreçlerinde etkin rol oynarlar (1). Tüm bu işlevler farklı potansiyele sahip kök hücreler tarafından idame ettirilir. Bu hücreler, bulundukları ortamda genelde uyku fazında olsa da, farklı sıklıkta kendilerini yeniler ve çevresel ve/veya içsel uyarılara cevaben farklılaşır ve çoğalırlar (2). Unipotent hücreler, sadece bulundukları doku hücrelerine farklılaşabilen hücrelerdir (3). Multipotent hücreler ise biraz daha geniş yelpazede farklılaşma kapasitesine sahip olsalar da farklılaşma kapasiteleri sınırlıdır (4). Birden fazla farklı doku sistemine farklılaşabilen hücreler ise pluripotent kök hücrelerdir (5). Bu hücrelerin ne şekilde ve hangi yöne doğru farklılaşacağını, bazı özel anatomik bölgelerde bulunan nörojenik niş hücreleri ve çevresel faktörler belirler (6). Yakın zamana

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kadar sinir sisteminin, özellikle de beyin gelişiminin belli bir sürede tamamlandığı ve bu süreç sonunda kendini yenilenme yeteneğinin ortadan kalktığı ileri sürülmüştür (7). Erişkinlerde nörogenez ve nörorejenerasyon olmadığı için de yeni sinir hücresi üretiminin olmadığı ve beyindeki ölen hücrelerin yerine yeni hücrelerin gelmediği düşünülmüştür (8). İlk kez, Altman tarafından 1962 yılında, nörogenezin, yetişkin sıçan beyninde hipokampusun dentat girusunda (DG) başladığı gösterildi (9). Devam eden çalışmalarda ise prenatal ve postnatal sıçan beyninde bölünebilen hücre varlığı, mitotik hücre bölünmesi sırasında eksternal olarak ortama konulan bir radyoaktif nükleozidin (3H-timidin) yeni kromozomal DNA zincirlerine dahil edilmesi temeline dayanan timidin katılım deneyi ile gösterildi (10,11). Ardından, nörogenez sırasında yeni oluşan hücrelerin beynin subventriküler bölgesinden (SVZ) olfaktor bulbusa (OB) doğru göç ettiği gösterilerek bulunan bu yolağa rostral göç akımı (RMS) adı verildi (12). Elde edilen bu bilgiler neticesinde, izole edilen multipotent hücrelerin kendini yenileme kabiliyeti olan nöral kök hücreler (NKH) olduğu kabul edildi (13).

Yetişkin Beynindeki Nörojenik Nişler

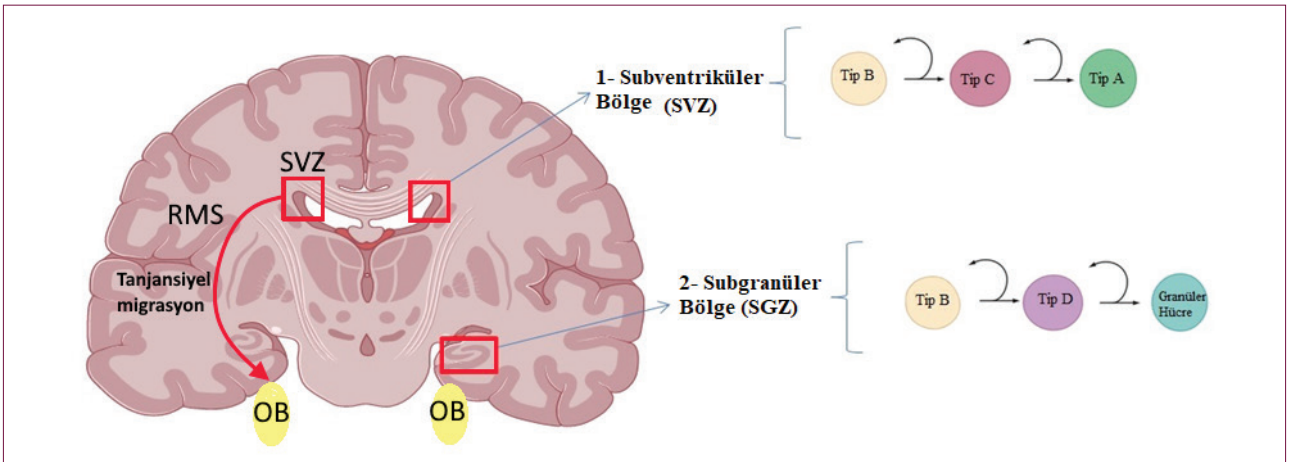
Yetişkin beyninde nörogenez sürecinde oluşan yeni nöronlar, beynin iki farklı bölgesindeki nişlerde bulunur. Bu iki anatomik bölge: (1) lateral ventrikülün SVZ ve (2) hipokampustaki DG'un subgranüler bölgesidir (SGZ) (14) (Şekil 1). Bu hücrelerin çoğu uyku fazında ve inaktif (quiescent) halde bulunan NKH'lerdir (15).

1. Subventriküler bölge (SVZ)

SVZ'deki proliferatif hücreler, lateral ventriküllerin dış duvarında bulunurlar. Bu hücreler, apikal yüzeylerindeki çıkıntılarıyla ventrikül lümenindeki ependimal hücrelerle doğrudan temas halinde olup glial fibriler asidik protein (GFAP) ve polisialize nöral hücre adhezyon molekülü (PSA-NCAM) proteinlerini eksprese ederler (16). SVZ'de bulunan NKH, dört farklı öncül hücreye farklılaşabilir. Bu hücreler: (1) nöral öncül hücreler, (2) oligodendrosit öncül hücreler, (3) astrosit öncül hücreler ve (4) glial sınırlı öncül hücrelerdir (17) (Şekil 2). Yetişkin fare beyninde, SVZ'de günde yaklaşık olarak 50.000 hücre üretilir (18). Bu hücrelerin yaklaşık %40'ı OB'ye ulaşır. Beyinde bu iki bölge arasındaki göç, 30 mikrometre/saat hızında gerçekleşir ve SVZ'den 5mm'lik bir yol katederek farklılaşacakları yer olan OB'ye doğru göç ederler (19). Bu migrasyon 5 -10 gün içerisinde önce tanjansiyel yönde sonra radyal doğrultuda gerçekleşir (20) (Şekil 1).

Yapılan çalışmalarda, RMS yolağındaki PSA-NCAM eksprese eden hücreler zincir şeklinde organize olurlar (21,22) ve göç eden nöroblastlar, OB'ye doğru giderken zincir şeklindeki yapısını korurlar. Nöron hücreleri, OB'ye doğru RMS aracılığıyla öncelikle tanjansiyel olarak göç ederler (12). Daha sonra immatür nöronlar, OB'nin daha dış katmanına radyal olarak hareket ederler. Astrositler, RMS aracılığıyla farklılaşacakları yer olan OB'ye doğru giderken immatür nöronlara farklılaşır. Bu hücreler granül hücreleri (%95) ve periglomerular nöronlar (%5) olarak adlandırılan iki tip inhibitör hücreye farklılaşırlar (23).

RMS sisteminde tip A (göç eden hücreler) ve tip B (astrositler) hücreleri olmak üzere iki ana hücre tipi vardır (24) . Tip A hücreleri sitoplazmalarında serbest ribozom ve mikrotübüllerden zengindir (25). Buna karşılık tip B hücrelerinin ise nukleus zarları daha polimorfiktir ve nükleuslarında bol olarak kondense heterokromatinler mevcuttur (26). Tip B hücreleri, memelilerin embriyonik dönemdeki ön beyninde bulunan temel NKH'lerdir (27). Bu hücrelerin radyal glial hücrelerden kaynaklandığına dair güçlü kanıtlar vardır. Tip B hücreleri asimetrik bölünerek iki yavru hücre oluşturur. Bunlardan biri parenteral hücreyle aynı olup diğeri daha kısa ömürlü olan ve bölünen



Şekil 1. Beyindeki nörojenik nişlerin şematik gösterimi: 1) SVZ' de bulunan tip B hücreleri asimetrik olarak mitozla bölünerek tip C hücrelerine ve parenteral hücrelere farklılaşır. Tip C hücreleri de tip A hücrelerine ve parenteral hücrelere farklılaşır. 2) SGZ' de bulunan tip B hücreleri asimetrik olarak mitozla bölünerek tip D hücrelerine ve parenteral hücrelere farklılaşır. Tip D hücreleri de immatür granüler hücrelere ve parenteral hücrelere farklılaşır. SVZ'de yer alan nöronlar olfaktör bulbusa doğru tanjansiyel olarak göç eder. Kısaltmalar: OB: Olfaktör Bulbus, SVZ: Subventriküler bölge, SGZ: Subgranüler bölge, RMS: Rostral göç akımı.

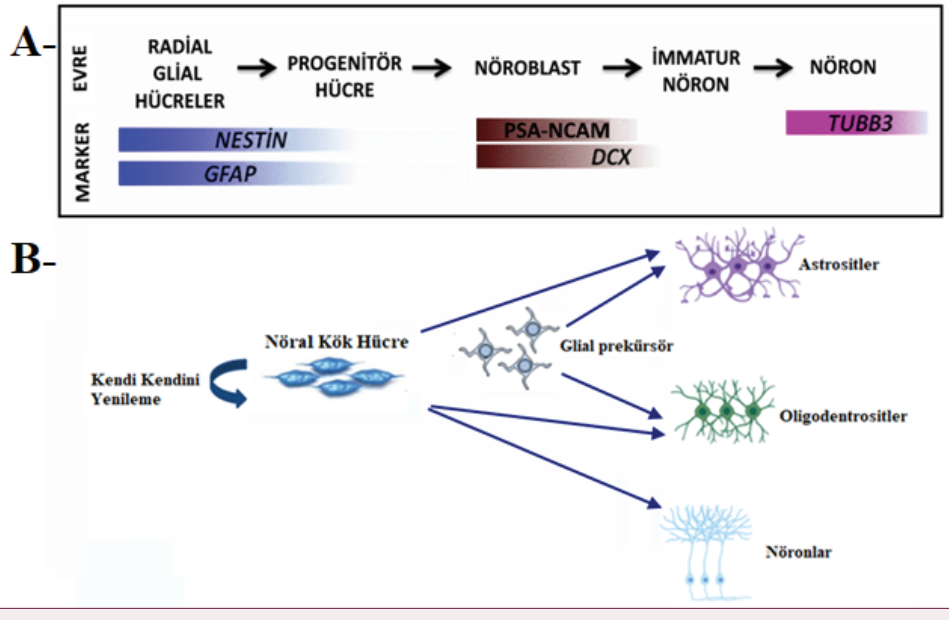
tip C (proliferatif öncüller veya nöroblast) hücreleri olarak adlandırılır (28,29). Tip C hücreleri antijenik olarak tip B hücrelerinden farklıdır (30). Tip C hücreleri göç eden nöroblastlar olan tip A hücrelerine farklılaşırlar (31) (Şekil 1). SVZ ve SGZ'deki tip C ve A hücreleri mikrotübül ilişkili protein olan doublekortin (*DCX*) ekspresyonuna bakılarak belirlenebilirler (32). Ayrıca *DCX* proteini, nörogenez seviyesini belirtmek için bir marker olarak kullanılabilir (Şekil 2A).

Tip A hücreleri membranları arasında yapısal karakteristik farklılıklar yaparak zonula adherens oluştururlar ve zincirler halinde düzenlenirler (33). Tip B hücreleri zincir şeklinde dizilmiş tip A hücrelerinin etrafını sarar. RMS sistemindeki bu zincir yapıları içerisinde tip A hücrelerinin gövde kısımları aktarılır (34). Zincir şeklindeki migrasyon çok sayıda nöral prekürsör kök hücrelerinin göç etmesine imkan sağlar. Zincir şeklinde aktarılan nöroblast hücreleri, çevrede bulunan parankimden salınan ekstrasellüler matriks proteini olan tenascin-R sayesinde izole bir ortamda bulunur (35). RMS'nin etrafını saran tip B hücreleri migrasyon için zinciri parankimden ayırarak göç için elverişli bir ortam oluşturur (36). Bu yapılara mikroglia hücreleri ve kılcal damarların yüzeyindeki endotel hücreleri eşlik eder (37). Bu yapılar kan damarlarının bazal laminası ve diğer astrositler ile gap juction yapar (38). Nöroblastlar, göç ettikleri yerde kendiliğinden diferansiyasyona uğrarlar ve mevcut nöral devreye kendilerini entegre ederler. Yeterli duyu girdisi alamayan entegre nöroblastların çoğu hayatta kalamamaktadır (39). Yapılan bazı çalışmalarda, yetişkin nörogenezindeki ependimal hücrelerin kemik morfogenetik proteini (Bone morphogenetic protein, BMP) antagonisti olan Noggin proteinini ürettiği gösterilmiştir (40). BMP'nin astrogenezi indüklediği, nörogenezi ise inhibe ettiği bilinmektedir. Ependimal hücrelerden üretilen Noggin'in nörojenik nişin korunmasında görevli olduğu düşünülmektedir (41). Bu etkileşimler NKH nişlerini düzenler ve astrositlerin ürettikleri sinyallerle *in vitro* çalışmalarda nörogenezin sürekliliğini sağlarlar (42).

2. Subgranüler Bölge (SGZ)

Yetişkin nörogenezinde ikinci niş olarak bilinen SGZ, hipokampal formasyonda bulunur (43). SGZ, hipokampus hilumuyla DG arasında uzanır. Beynin bu bölgesinde tıpkı SVZ'de olduğu gibi yeni nöronların primer prekürsörlerini astrositler oluşturur. SGZ'de bulunan astrositler tip B hücreleri olarak adlandırılır (44). Ancak bu bölgedeki hücreler SVZ'deki tip B hücrelerinden hücresel yapı olarak farklıdırlar. Hipokampustaki tip B hücreleri immatür tip D hücrelerine farklılaşır ve bunların SVZ'deki tip C hücreleri gibi işlevi vardır (45).

Tip D hücreleri hipokampusun granüller tabakasına göç ederek uyarıcı hücrelere farklılaşırlar (46). SGZ nişindeki NKH'ler, SVZ nişindeki hücrelerle karşılaştırıldıklarında parenteral hücrelere daha çok benzerlik gösterirler. Daha sonra bu farklılaşan hücreler öğrenme ve hafızayla ilişkili bölge olan hipokampusun CA3 bölgesine mossy fibrilleri olarak isimlendirilen aksonal



Şekil 2. Nöral kök hücrelerin farklılaşması süresince tespit edilen gen anlatım değişiklikleri ve oluşan nöral hücrelerin şematik gösterimi. A) NKH in farklılaşma evreleri olarak belirlenmiş olan radial glial hücre aşaması, progenitor hücre aşaması, nöroblast, immatür nöron ve nöron evrelerini içerir. Her bir evre tespit edilen artmış gen anlatım ifadeleri ve evre sonunda azalan gen anlatımı farklı renklerle gösterilmiştir. Sırasıyla radial glial hücre aşamasında artan gen anlatımı *NESTİN ve GFAP* için mavi renk, nöroblast evresinde *DCX* geni için kahve renk ve nöron evresinde *TUBB3* geni için pembe renk içermektedir. Gen anlatım azalışı ise, her bir gen için belirlenen rengin solması şeklinde gösterilmiştir. B) NKH'nin farklılaşma süresinde göstermiş olduğu hiyerarşik düzende NKH'ler farklılaştıklarında astrositler, oligodentrositler ve nöronları oluşturur.

projeksiyonlarla uzanırlar (47). Hipokampustaki nöronal devreye entegre olan NKH'ler CA3 piramidal hücrelere ve internöron hücrelerine farklılaşırlar (48). SGZ'deki astrositler diğer NKH nişi olan SVZ'deki hücrelerden farklı olarak PSA-NCAM eksprese etmezler. Hipokampustaki nörogenez sürecini yaş, uyku ve stres gibi faktörler etkiler (49).

Yetişkinde, nörojenik niş özelliği olmayan korteks, spinal kord ve serebellumdaki astrositlerin, doğumdan sonraki ilk iki hafta içerisinde nörojenik özellik gösterdiği *in vitro* çalışmalarda gösterilmiştir (50). İnsan beyninde bazı bölgelerin nörojenik özelliği vardır. Ancak beynin ve spinal kordun birçok kısmı gliojenik özellik gösterir (51). SVZ'de transkripsiyon faktörü olan Oligodendrosit transkripsiyon faktörü 2 (Oligodendrocyte Transcription Factor 2, *OLIG2*), aynı zamanda gliogenezde önemli bir moleküler belirteçtir. *OLIG2*, nörogenezin durduğunu gösterir ve aynı zamanda glial hücrelerin kaderini belirler (52).

Büyüme Faktörlerinin Nörogenez Üzerindeki Etkileri

Yetişkin nörojenik nişlerde sessiz halde bulunan NKH'ler nörotrofik faktörler aracılığıyla aktif hale gelebilirler. Bu büyüme faktörleri niş içerisinde hücre-hücre ve hücre-ekstrasellüler matriks etkileşimini uyarırlar (53). Büyüme faktörleri temelde niş içerisindeki hücrelerden veya dış kaynaklardan salınıp beyin omurilik sıvısı (BOS) ve kan damarları yardımıyla ilgili anatomik bölgeye ulaşırlar. Yapılan çalışmalarda Fibroblast büyüme faktörü (FGF), epidermal büyüme faktörü (EGF) ve transforme edici büyüme faktörü alfa (TGF-α) gibi büyüme faktörlerinin koroid pleksustan BOS'a salınarak, subependimal bölgedeki (SEZ) hücre proliferasyonu üzerinde etkisi olduğu gösterilmiştir (54). Bu büyüme faktörleri, NKH nişleri için spesifik değildir. Ancak tüm kök hücre nişlerinde proliferasyon süreçlerinde görev alırlar (55).

Vasküler endotelial büyüme faktörü (VEGF) uyarımı, NKH'lerin proliferasyonu, hayatta kalması ve nöral progenitör hücrelerin göçünde ve olgunlaşmasında önemli bir rol oynar (56). VEGF hem anjiogenez hem de nörogenezde rol alan büyüme faktörüdür (57). Bu anjiogenik etkisini kapiller hücreler üzerinden gösterirken, nörojenik etkisini ise kapiller hücre komşuluğundaki endotel hücreler üzerinden gösterir. VEGF, endotel hücrelerinden nörotrofin 3 (NT3) salgılatarak sessiz haldeki NKH'lerin farklılaşması üzerine etki eder (58,59). NKH ve progenitörlerin proliferasyonunu düzenleyen VEGF, Trombosit kökenli büyüme faktörü (PDGF) gibi bir dizi vaskülariteye bağlı büyüme faktörleri tanımlanmıştır (60). VEGF'nin hem *in vitro* hem de intraventriküler uygulanması sonucunda SEZ' de ve SGZ'de yer alan progenitör hücrelerin proliferasyonunu artırdığı gösterilmiştir (61). NKH'ler PDGF alfa reseptörünü eksprese eder. Bu ekspresyon seviyeleri nöron ve oligodentrosit son ürünleri arasındaki dengeyi sağlar (62). SEZ'deki NKH'lerde reseptör defekti nörogenezi etkilemezken, oligodendrosit üretiminde bir azalmaya neden olur. PDGF'nin lateral ventriküllere infüzyonu tip-B hücre üretimini arttırdığı ve nöroblast üretimini engellediği gözlemlenmiştir (63).

Beyin kökenli nörotrofik faktör (BDNF), yetişkin nörogenezinde nöral progenitör hücre proliferasyonunu, farklılaşmasını ve hayatta kalmasını teşvik etmek için endotel hücreleri tarafından salgılanmaktadır (64). Yapılan ko-kültür deneyleri hücrelerin BDNF salgılamasının nörogenezi desteklediği gösterilmiştir (65). SVZ'de nöroblastlar gama amino bütirik asit (GABA) salgılar, hücre dışı BDNF'yi yakalamak için astrositlerde tropomiyosin reseptör kinaz B (Tropomyosin-Related Kinase B, *TRKB*) ekspresyonunu indükler. Bu durum RMS'de nöroblast göçünü uyarır (66). Eritropoietin (EPO) ise NKH proliferasyonunu ve nörosferlerin nöroblast şeklinde farklılaşmasını artırır (67).

İnsülin benzeri büyüme faktörü (IGF-I), hem *in vitro* hem de *in vivo* NKH proliferasyonunu büyük ölçüde artıran mitojenik bir faktördür (68). IGF/İnsülin benzeri büyüme faktörü 1 reseptörü (IGFR) sistemi, embriyo ve yetişkin beyinlerinde NKH'lerden üretilen nöronların, astrositlerin ve oligodendrositlerin farklılaşmasını düzenler (69). IGF-I ile yapılan önceki çalışmalarda apoptozu, reaktif oksijen türleri (ROS) aşırı üretimini ve DNA hasarını inhibe ederek hücrelerin hayatta kalmasını desteklediği gösterilmiştir (70,71). Bu bulgu, IGF'nin sinir hasarından sonra nörogenez için çok önemli bir nöroprotektif etkiye sahip olduğunu göstermektedir (72). IGFs ve IGF-I reseptörlerindeki mutasyonların mutant farelerin hem beyin gelişiminde hem de vücudun diğer kısımlarında önemli eksiklikler oluşturduğu gözlemlenmiştir. Yapılan çalışmalarda, fare embriyonik strial NKH proliferasyonu üzerinde IGF-I 'in EGF ve FGF-2 ile sinerjistik etkiye sahip olduğu gösterilmiştir (73,74) . IGF-I'in NKH'lerde otokrin salgılanması IGF-I ve EGF üzerinde ko-stimülasyon oluşturur (75). Gelişim sürecinde EGF varlığının organogenezde beyin hacmini arttırıcı etkisi olduğu görülmüştür (76). SVZ'de bulunan kök hücreler EGF ve FGF reseptörlerini eksprese eder (77). Hücrelerin bu iki faktörle uyarılması *in vitro* olarak NKH'lerin proliferasyonunu indükleyebilir. EGF ayrıca tip C hücrelerine etki ederek multipotent kök hücrelere farklılaşmalarını ve nöroblast üretimini azaltır (78). Bu iki büyüme faktörünün *in vivo* uygulaması, NKH'lerin çoğalması ve farklılaşması için önemlidir. EGF ve FGF-2 nörotrofik faktörlerin kombinasyonu, hem hipokampusun dentat girusunda hem de sıçan beyinlerinde SVZ'sinde progenitör hücre proliferasyonunu indükler (79).

FGF-2, yetişkin nörogenezinin diğer önemli endotel kökenli efektörüdür. FGF-2, SVZ ve hipokampusun dentat girusundaki granül hücre progenitörlerinin yenilenmesi ve proliferasyonunu düzenler (80). Yetişkin merkezi sinir sisteminde (MSS) FGF-2, nörojenik nişlerde eksprese edilir (81). Yetişkin nöral kök ve progenitör hücrelerin proliferasyonundaki ve farklılaşmasındaki değişikliklere bağlı olarak FGF-2 yetişkin nörogenezinin kontrolünde rol oynar. SVZ'de, FGF-2'nin GFAP- pozitif hücreler tarafından yüksek oranda eksprese edildiği gösterilmiştir. Ancak nestin-pozitif olgunlaşmamış nöronlar tarafından eksprese edilmez (82 ,83). Astrositik FGF2, akut stresi takiben SGZ' de artmış nörogenez ile ilişkilendirilmiştir. SVZ ve SGZ'deki NKH'ler de yüksek oranda eksprese edilen FGF-2 reseptörlerinden biri olan FGFR1, hipokampal NKH proliferasyonu için gereklidir (84). FGF-2, NKH'nin yayılmasını hem *in vitro* hem de *in vivo* olarak düzenler. Yapılan çalışmalar, FGF2'nin nörogenezde, gelişim sırasında ve yetişkin beyninde hem kök hücrelerin proliferasyonu hem de farklılaşmasındaki önemli rolünü göstermiştir (85).

FGF2/ FGFR1 sinyali, sadece nörogenez üzerinde değil, sinaptik formasyon, nöron-glia etkileşimleri, inflamasyon ve amiloidoz üzerindeki yerleşik etkileri nedeniyle MSS hastalıklarına yönelik terapötik müdahaleler için büyük umut vadetmektedir.

FGF-2, EGF ve IGF-1 tek başına uygulandığında NKH'ler üzerinde mitojenik etki göstermezken kombinasyonlar halinde uygulandığında NKH'lerin proliferasyonu ve hayatta kalması üzerinde pozitif etkiye sahip olduğu gösterilmiştir (86).

Nörodejeneratif Hastalıklar ve Büyüme Faktörleri

Nörodejeneratif hastalıklar, merkezi sinir sistemi veya periferik sinir sistemindeki yapısal ve işlevsel bozukluklar ile karakterize edilen heterojen bir gruptur (87). Nörodejeneratif hastalıkların birçoğunda nöronal ve sinaptik kayıp, bilişsel bozukluk ve enfeksiyon gibi durumlar görülür. Parkinson hastalığı (PH), Alzheimer hastalığı (AH), Huntington hastalığı (HH), Şizofreni ve Prion hastalığı nörodejeneratif hastalıklar arasında yer alır (88). Yetişkin nörogenezindeki değişiklikler AH, PH, ve HH'nin dahil olduğu farklı nörodejeneratif hastalıklarda ortak olarak görülür. Çünkü farklı hastalıklardan sorumlu olan farklı patolojik proteinler, farklı sinir popülasyonlarının kaybına neden olur (89).

Parkinson hastalığı, hipokampusu ve OB'yi erken dönemde etkiler. Bu durum patolojik olarak hücre içi α-sinüklein birikintileri ve nöronların dejenerasyonu ile karakterizedir (90). Alzheimer hastalığı, bazal ön beyin ve limbik sistemdeki dejeneratif hastalıklardır. Bu durum patolojik olarak nörofibriler ve amiloid plaklarla karakterize edilir (91). Huntington hastalığında, otozomal dominant bir mutasyona bağlı olarak Huntington geninde trinükleotid artışı olur. Bu durum NKH'lerin çoğalmasını etkilemez ancak nöronların olgunlaşmasında bozulmalara neden olur (92). Şizofreni, klinik olarak halüsinasyonlar, düşünce bozukluğu ve hareket bozukluğu ile karakterize edilen başka bir yaygın nörodejeneratif hastalıktır (93). Glutamat ve dopamin sistemleri nöronal sinyalleşme açısından farklı roller oynarlar, ancak her ikisinin de şizofreni patofizyolojisine önemli ölçüde katkıda bulunduğu öne sürülmüştür (94). Araştırmacılar, nörotrofik temelli tedavilerin nörodejeneratif hastalıkların tedavisinde etkili olabileceğini düşünmektedir (95). Tablo 1'de bu çalışmaların bir özeti sunulmaktadır. Nörotrofik faktörler, sitokinler ve kemokinler, epigenetik faktörler ve sinyal yolakları gibi çoklu modülatörler, yeni oluşan nöronların oluşumunu etkiler (96). Her modülatör, NKH çoğalmasını, farklılaşmasını, göçünü ve hayatta kalmasını farklı şekillerde yönlendirir. Ancak, uzun yıllar beyin hasarı ve hastalıklarında hasarlı bölgedeki nöronların rejenere olamayacağı genel kabul gören bir gerçektir.

NGF, BDNF, gliadan türetilmiş sinir faktörü (GDNF) ve IGF-I içeren endojen nörotrofik büyüme faktörleri, NKH proliferasyonunu, farklılaşmasını ve MSS'ni uyarmada önemli bir role sahiptir (97). Hücresel düzeyde birçok nörotrofik faktörün, NKH'lerin kendi kendini yenilemesi ve olgunlaşması üzerinde etkileri vardır. Nörotrofik faktörler tropomiyozin ile ilişkili kinaz (Trk) reseptörlerinin aktivasyonunu uyarır. BDNF-TrkB sinyallemesinin, hipokampal nörogenezin upregülasyonunda ve yetişkin nörogenezi sırasında yeni doğan nöronların hayatta kalmasında önemli olduğu gösterilmiştir (98). Nörodejeneratif hayvan modellerinde tedavi amaçlı NKH uygulamasıyla NGF ve BDNF gibi nörotrofik faktörlerin aşırı ekspresyonu inflamasyonu azaltarak nöronları dejeneratif süreçten koruduğu gösterilmiştir (99). Bu durumun PH, HH, AH gibi nörodejeneratif hastalıkların tedavisinde etkili olabileceği düşünülmektedir. Bir başka çalışmada da bozulmuş nörogenezin hem AH hem de PH için erken bir klinik belirteç olduğu düşünülmektedir (100). Bu nedenle düşük nörotrofik faktör seviyeleri nörogenezdeki bozulmaların habercisi olabilir. BDNF ve NGF'nin yetişkin nörogenezin sürdürülmesindeki rolleri nedeniyle AH ve PH için bu nörotrofik faktörlerin artışları terapötik fayda sağlayabilir (101,102).

Mekanik büyüme faktörü (MGF) ile yapılan bir çalışmada hipokampus ve OB'de bulunan NKH popülasyonunu artırarak nörogenez üzerinde etkisi olduğu gösterilmiştir. Elde edilen verilerde MGF'nin, nöral progenitör hücrelerin korunmasını ya da proliferasyonunu sağlayarak NKH'leri arttırdığı gösterilmiştir (103).

Angionorinlerin, nörovasküler çapraz iletişimi düzenleyerek hem nöral hem de vasküler hücre sürecini etkilediği kabul edilmiştir (104). Duygu durum bozukluğu olan hastalarda VEGF, FGF-2, NGF ve IGF-I seviyeleri hakkında yetersiz ve tutarsız araş-

Tablo 1. Nörodejeneratif hastalıkların tedavisinde kullanılabilecek potansiyel nörotrofik faktörler.

Nörodejeneratif Hastalıklar	Büyüme Faktörleri	Hedef Nöronlar	Tedavi	Referans
Alzheimer Hastalığı	IGF-1, BDNF, NGF, GDNF, FGF,	Striatal Nöron	Faz 1 çalışması tamamlandı. Faz 2 çalışmaları devam ediyor (NGF and BDNF)	80,106–110
Parkinson Hastalığı	IGF-1, BDNF, FGF, CDNF, GDNF	Kolinerjik Nöron, Entorhinal Nöron	Faz 1 çalışmaları devam ediyor (GDNF/Neurturin)	111–115
Huntington Hastalığı	BDNF, NGF, FGF, CNTF	Striatal Nöron	Preklinik çalışmalar (BDNF)	116–119
Şizofreni	BDNF, NGF	Dopaminerjik hücreler	Biomarker olarak (BDNF)	120–122

tırmalar vardır (105). Yapılan bir çalışmada VEGF, FGF-2, NGF ve IGF-1'in serum seviyeleri ile bipolar bozukluğun manik dönemi arasındaki ilişki araştırılmıştır. Bulgular NGF, FGF-2 ve IGF-1'in bipolar bozukluğun patofizyolojisi ile ilgili olabileceğini düşündürmektedir. Ancak daha fazla çalışma gerekmektedir.

Nörodejeneratif hastalıkların yanı sıra, ağrı, anksiyete, depresyon, obezite ve diğer durumlarda da nörotrofik faktörlerin seviyelerinde değişiklikler görülür. Örneğin BDNF, psikiyatrik bozukluklarla ilişkili nöronal ağların oluşumunu ve sürdürülmesini düzenler. Serumda BDNF seviyesinin azalması depresyon ile ilişkilendirilir. Anti-depresan tedavisi ile serumda bulunan BDNF'nin seviyesinde artış görülür (123). Ek olarak, serumda BDNF'nin daha düşük seviyelerde görülmesi yetişkinlerde hipokampal hacim azalması ve hafıza geriliği ile ilişkilendirilmiştir. Serumda yüksek BDNF seviyelerinin ise bireylerde demansa karşı koruyucu etkiye sahip olduğu bulunmuştur (124). Nörotrofin sinyallenmesi birçok nörodejeneratif ve psikiyatrik bozukluk için geçerli olduğundan, nörotrofin düzeylerini artırmak depresyon gibi psikiyatrik bozuklukları, PH ve AH gibi nörodejeneratif hastalıkları iyileştirmek için yeni tedaviler sağlayabilir. Son olarak, beyindeki nörotransmiterlerin ve hormonal aktivitenin manipülasyonu, terapötik faydalar için yetişkin nörogenezini güçlendirmede de yararlı olabilir. Gelecekteki çalışmalar nörotrofik faktörlerin, yaşlanma ve nörodejeneratif hastalıklar için klinik uygulanabilirliğini ortaya çıkaracaktır.

SONUÇ

Nörojenik mekanizmalar ve yetişkin nörogenezini belirleyen faktörler hakkındaki bilgiler, son birkaç yılda büyük mesafe katetmiştir. Halen, nörotrofik faktörlerin, yetişkin nörogenezi üzerinde kontrol edici ve düzenleyici etkilerini belirlemek ve yetişkin beyninin nörojenik alanlarını karakterize etmek amacıyla kapsamlı çalışmalar yapılmaktadır. Heyecan verici bir konu olmasının nedeni özellikle yeni oluşan nöronların yetişkin beyninde fonksiyonel aktivitedeki rolleri ve bu hücrelerin klinik yansımasıdır. Buna ek olarak, nörodejeneratif hastalıkların tedavi yaklaşımında öncelikle, merkezi sinir sistemindeki özel bölgelerde yer alan büyüme faktörlerinin seviyelerinin nasıl arttırılacağı ve bu faktörlerin dışarıdan uygulanması durumunda en etkin aktivitenin nasıl elde edileceği önemlidir. Bazı çalışmalar, büyüme faktörlerinin *in vivo* olarak kan beyin bariyerini (KBB) geçemediğini gösterirken (125), bir başka çalışma KBB'yi geçme yeteneğinin var olabileceğini (126), ancak söz konusu geçirgenliğin büyüme faktörüne bağlı olarak farklılık göstereceğini bildirmektedir.

Teknolojide yaşanan son yenilikler araştırma alanı ile birleşince birçok hastalığın tanı ve tedavisinde başarılı sonuçlar elde edilmektedir. Bu bağlamda, özellikle nörodejeneratif hastalıklarda kullanılabilecek büyüme faktörlerinin yeni tedavi yaklaşımlarıyla da desteklenmesi kat edilen başarıyı daha da ileriye taşıyacaktır. Eksternal uygulanan ilaçların, beyin parankimine girmesinde büyük engel oluşturan KBB (127) viral vektörler (128), hücre bazlı kanallar (129), polimer kapsülleme (130), geliştirilmiş farmakokinetik uygulamalarla artırılan beyin geçirgenliği ile peptid taklit edilmesi (131), küçük molekül replikasyonları (132) gibi araçlar ile aşılmaya çalışılmaktadır. Lipozomlar, çeşitli maddeleri kapsülleyebilen nano taşıyıcılardır (133). Lipozomların yüzeyleri siklik peptidle modifiye edilerek KBB'yi geçmeleri için kullanılmaktadır (134). Dolayısıyla büyüme faktörlerinin sistemik olarak kullanılmalarındaki en büyük engel olan KBB'yi aşabilmek için lipozom vektörlerle kombinasyonları kullanılabilir. Örneğin, KBB'yi geçemeyen ve sistemik uygulamayı takiben etkili nörofarmasötik ajan özelliği olmayan nörotrofinler, çeşitli vektörlere konjuge edilerek KBB'yi aşabilir ve hedeflenen bölgeye gitmeleri sağlanabilir (135). Tüm bunların yanında, intranazal ilaç uygulamaları da KBB'yi aşmada yüksek başarı oranına sahiptir ve ilacın MSS'ye hızlı ulaşımını sağlar (136). İlaç uygulamasının çok pratik olması, dozun kolayca tekrarlanabilir olması ve ilaç modifikasyonuna gerek olmaması gibi avantajları ile bu uygulama klinik potansiyele sahiptir. Nörotrofinlerin bu kombinasyonlarının intranazal yolla uygulanabilmesi ile doğru zamanda etkin süreyle kullanımı sonucu nörorejenerasyonu uyararak nörodejeneratif hastalıklarda tedaviye fayda sağlayabilir. Bu ve benzeri faktörlerin terapötik uygulanmalarıyla ilgili problemler ilerleyen çalışmalarda daha netlik kazanacaktır.

Yukarda bahsedildiği üzere, yakın zamanda özellikle NKH'lerin karakter ve fonksiyonel özelliklerini anlamada kat edilen mesafe, nöro-rejenerasyonun sanıldığının aksine indüklenebilir ve kontrol edilebilir olduğunu göstermiştir. Yetişkin nörogenezde büyüme faktörlerinin eksojen olarak kullanılmasıyla dejenere olan nöronların, rejeneratif sürece girebildiği deneysel platformlarda gösterilmiştir. Ancak nörodejeneratif hastalıkların tedavisinde etkin bir kullanımı henüz mevcut değildir. Günümüzde nörotrofik faktörlerin beyne taşınması, kök hücre ve gen terapileri ile sağlanmıştır. Sonuç olarak büyüme faktörlerinin terapötik ajan olarak kullanımı, yetişkin nörogenezinin devamlılığı için etkin olabilir. Gelecekteki çalışmalarda bu faktörlerin tedavide kullanımı, yaşlanmanın önlenmesine ve nörodejeneratif hastalıkların mekanizmalarının anlaşılmasına önemli bir katkı sağlayacak ve yeni tedavi yöntemlerinin geliştirilmesine ışık tutacaktır.

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EXPERIMED

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

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

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

References

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

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

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

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

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

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

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

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

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

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

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

REVISIONS

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

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

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EXPERIMED

YAZARLARA BİLGİ

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

Experimed, temel ve klinik tıp bilimlerinin tüm alanlarında orijinal araştırma, olgu sunumu, derleme ve editöre mektup türlerinde makaleler yayınlamaktadır.

Yayın Politikası
Derginin editöryel ve yayın süreçleri International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), ve National Information Standards Organization (NISO) organizasyonlarının kılavuzlarına uygun olarak biçimlendirilmiştir. Experimed'in editöryel ve yayın süreçleri, Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice) ilkelerine uygun olarak yürütülmektedir.

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

EXPERIMED

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 Experimed Dergisi'nde, yayınlanan çalışmalarının telif hakkına sahiptirler ve çalışmaları Creative Commons Atıf-GayrıTicari 4.0 Uluslararası (CC BY-NC 4.0) olarak lisanslıdır. Creative Commons Atıf-GayrıTicari 4.0 Uluslararası (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.

Yazarlar, basılı ya da elektronik formatta yer alan resimler, tablolar ya da diğer her türlü içerik dahil daha önce yayınlanmış içeriği kullanırken telif hakkı sahibinden izin almalılardır. Bu konudaki yasal, mali ve cezai sorumluluk yazarlara aittir.

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 ta-

rafından 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 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.

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

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.

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

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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/ncidodIEID/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.

Yayına kabul edilen makaleler dil bilgisi, noktalama ve biçim açısından kontrol edilir. Yayın süreci tamamlanan makaleler, yayın planına dahil edildikleri sayıyla birlikte yayınlanmadan önce erken çevrimiçi formatında dergi web sitesinde yayına alınır. Kabul edilen makalelerin baskıya hazır PDF dosyaları sorumlu yazarlara iletilir ve yayın onaylarının 2 gün içerisinde dergiye iletilmesi istenir.

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