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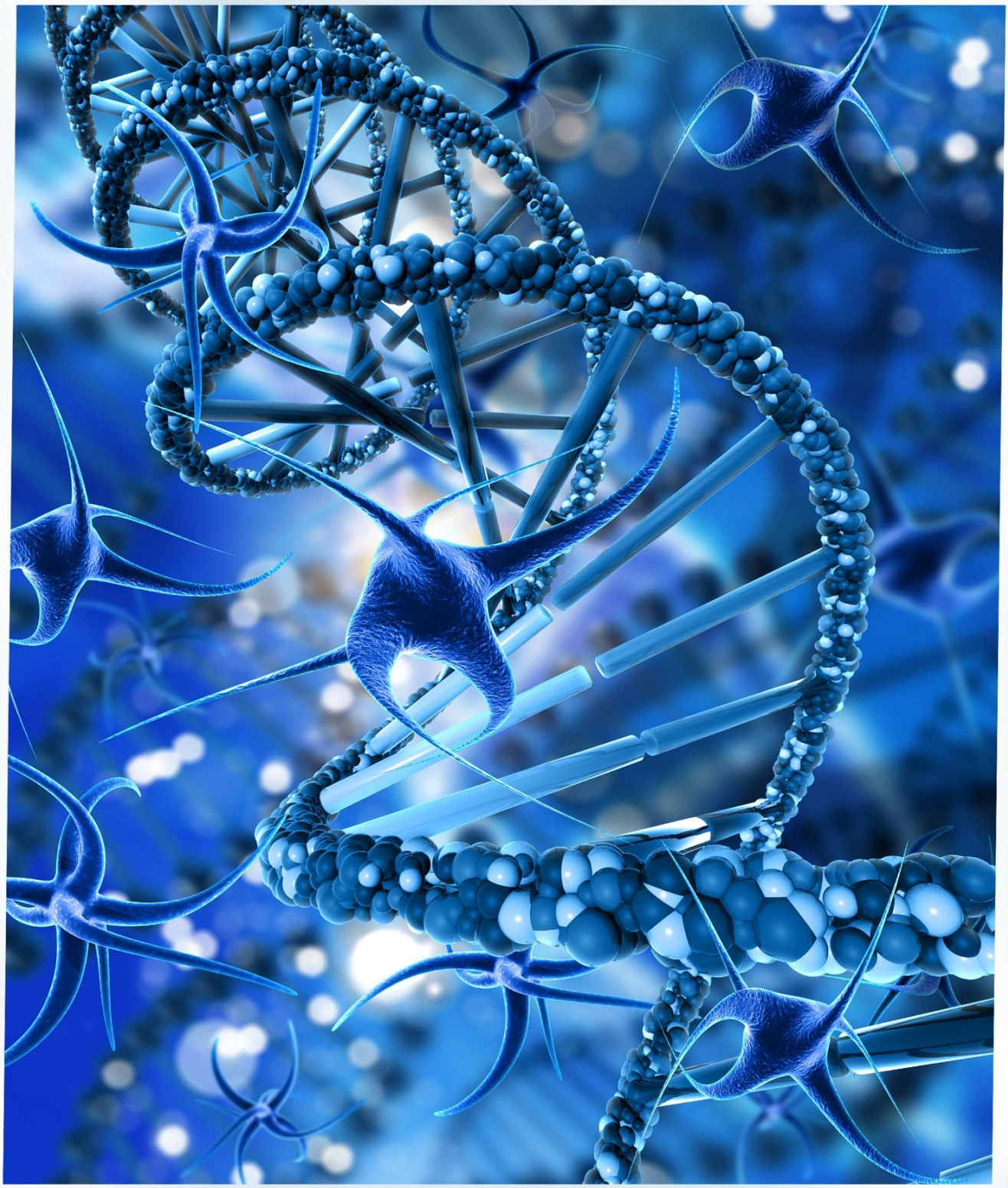

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Therapeutic Effect of Sinapic Acid against 5-Fluorouracil-Induced Oxidative Stress and Inflammation in Rat Ovarium: An Experimental Approach

Sıçan Yumurtalık Dokusunda 5-Florourasil ile Uyarılan Oksidatif Stres ve İnflamasyona Karşı Sinapik Asitin Terapötik Etkisi: Deneysel Bir Yaklaşım

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ABSTRACT

Tissue toxicity caused by 5-fluorouracil (5-FU) is associated with increased reactive oxygen species and inflammatory cytokines. Sinapic acid (SA) has both antioxidant and anti-inflammatory activities. Although SA has been shown to ameliorate chemical-induced tissue damage in various experimental models, its effects against 5-FU-induced ovarian damage have not yet been investigated. It was therefore aimed to evaluate the therapeutic potential of SA against 5-FU-induced ovarian damage in rats, together with the mechanisms of oxidative stress and inflammation in this study for the first time. Thirty rats were distributed into five groups: control, 5-FU (100 mg/kg) 5-FU+SA (2.5 and 5 mg/kg) and SA (5 mg/kg). 5-FU was applied to rats intraperitoneally on the 1st day of experiments and then SA was administrated for 3 consecutive days. The levels of lipid peroxidation [malondialdehyde (MDA)], oxidative stress (total oxidant status (TOS) and oxidative stress index (OSI)], antioxidant system [total antioxidant status (TAS), and catalase (CAT)], DNA damage [8-hydroxy-2'-deoxyguanosine (8-OHdG)] and inflammatory [interleukin-6 (IL-6)] markers in ovarian tissues were determined using spectrophotometric methods. It was determined that a single dose of 5-FU administration in rats significantly increased oxidative stress and inflammation in the ovarian tissue and suppressed the antioxidant system compared to the control group ($p<0.05$). It was revealed that SA significantly suppressed ovarian inflammation by decreasing IL-6 levels, attenuates DNA damage by decreasing 8-OHdG levels, and also provides restoration of oxidative stress by decreasing MDA, TOS and OSI levels and increasing TAS and CAT levels in a dose-dependent manner. In conclusion, we found that SA exhibits therapeutic effects against 5-FU-induced ovarian damage. These findings suggest that SA may be a potentially useful agent for protection against chemotherapeutic-induced ovarian injury.

Keywords: 5-fluorouracil, Inflammation, Ovarian damage, Oxidative stress, Rat, Sinapic acid

ÖZET

5-florourasil (5-FU)'nun neden olduğu doku toksisitesi, artan reaktif oksijen türleri ve inflamatuar sitokinler ile ilişkilidir. Sinapik asit (SA) bir fenolik asittir ve hem antioksidan hem de anti-inflamatuar aktivitelere sahiptir. Çeşitli deneysel modellerde SA'nın kimyasal kaynaklı doku hasarını iyileştirdiği gösterilmiş olmasına rağmen, 5-FU kaynaklı yumurtalık hasarına karşı etkileri henüz araştırılmamıştır. Bu nedenle bu çalışmada SA'nın sıçanlarda 5-FU ile indüklenen yumurtalık hasarına karşı terapötik potansiyelinin oksidatif stres ve inflamasyon mekanizmaları ile birlikte ilk kez değerlendirilmesi amaçlandı. Otuz sıçan beş gruba ayrıldı: kontrol, 5-FU (100 mg/kg), 5-FU+SA (2,5 ve 5 mg/kg) ve SA (5 mg/kg). Sıçanlara deneylerin 1. günü intraperitoneal yoldan 5-FU, ardından 3 gün boyunca SA uygulandı. Yumurtalık lipid peroksidasyon seviyeleri [malondialdehit (MDA)], oksidatif stres (toplam oksidan durum (TOS) ve oksidatif stres indeksi (OSI)], antioksidan sistem [toplam antioksidan durum (TAS) ve katalaz (CAT)], DNA hasarı [8-hidroksi-2'-deoksiguanozin (8-OHdG)] ve inflamatuvar [interlökin-6 (IL-6)] belirteçlerinin düzeyleri spektrofotometrik yöntemlerle belirlendi. Sıçanlarda tek doz 5-FU uygulamasının kontrol grubuna göre yumurtalık dokusunda oksidatif stresi ve inflamasyonu anlamlı olarak arttırdığı ve antioksidan sistemi baskıladığı belirlendi ($p<0.05$). SA'nın IL-6 düzeylerini düşürerek over inflamasyonunu önemli ölçüde baskıladığı, 8-OHdG düzeylerini düşürerek DNA hasarını azalttığı ve ayrıca MDA, TOS ile OSI düzeylerini düşürerek ve TAS ile CAT düzeylerini artırarak oksidatif stresin restorasyonunu doza bağımlı bir şekilde sağladığı ortaya konuldu ($p<0.05$). Sonuç olarak, SA'nın 5-FU ile indüklenen yumurtalık hasarına karşı terapötik etkiler gösterdiğini bulduk. Bu bulgular SA'nın kemoterapötik kaynaklı yumurtalık hasarına karşı koruma için potansiyel olarak yararlı bir ajan olabileceğini düşündürmektedir.

Anahtar Kelimeler: 5-florourasil, İnflamasyon, Oksidatif stress, Rat, Sinapik asit, Yumurtalık hasarı

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INTRODUCTION

Cancer is the leading cause of death worldwide.[1] Although chemotherapy is one of the most widely used treatments to treat various types of cancer, the cytotoxic effect of chemotherapeutic drugs on rapidly proliferating normal cells remains an unsolved problem.[2] With the increasing improvements in cancer survival rates with chemotherapy, reducing the significant long-term side effects that affect patients following treatment is critical. For women, damage from chemotherapy to the reproductive system results in premature menopause and/or infertility.[3] 5-fluorouracil (5-FU) is a widely used anti-neoplastic agent against a variety of tumors, including carcinomas of the gastrointestinal tract, breast, head and neck.[4] 5-FU exhibits its effect by inhibiting RNA and DNA synthesis in rapidly dividing cells, including cancer cells.[3] The anticancer effect of 5-FU is due to its ability to inhibit the enzyme thymidylate synthase and to form intermediates, such as fluorouridine triphosphate and fluorodeoxyuridine triphosphate, that are incorporated into RNA and DNA. All these mechanisms suppress DNA repair mechanisms that lead to cell death.[4] However, 5-FU treatment can lead to significant toxic conditions resulting in myelotoxicity, neurotoxicity, cardiotoxicity and mucositis.[3] Although information on reproductive toxicity is limited since 5-FU is usually administered with other chemotherapeutic drugs, it has been shown that 5-FU administration causes ovarian damage in experimental animals in recent years.[5-7] It is well documented that overproduction of reactive oxygen species (ROS) and inflammatory mediators play a vital role in the toxic manifestations induced by 5-FU. Therefore, it is suggested that the use of agents with antioxidant and anti-inflammatory potential may be beneficial in eliminating 5-FU-related tissue toxicity.[2]

The phytochemicals, found in plants, are reported to have numerous benefits in the treatment of cancer, liver ailments, diabetes, heart diseases, kidney ailments, and many more. The use of plant-based products has therefore increased significantly all over the world.[8] Phenolic compounds have attracted the attention of researchers as their powerful antioxidant properties can protect the body against free radicals and oxidative stress in recent years.[9] Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SA) is a natural herbal compound included in the phenolic acid group. It is found in various natural products, such as wheat, rice, spices, oil seeds, vegetables, citrus fruits, cereals and vinegar.[10,11] Antioxidant, anti-inflammatory, anticancer, antihyperglycemic, antidiabetic, hepatoprotective, cardioprotective, renoprotective, neuroprotective, anxiolytic and antibacterial activities of SA have been demonstrated in previous studies.[8,10] The tissue protective role of SA against dimethylnitrosamine, cisplatin, bleomycin and cyclophosphamide induced injury have also been previously reported.[9,11-13] Therefore, we hypothesized that SA could effectively suppress 5-FU-induced ovotoxicity and sought to explore the therapeutic efficacy of SA against 5-FU-induced ovarian damage and to uncover the mechanisms underlying this in the current research for the first time.

METHODS

Chemicals and kits

Phosphate buffered saline (PBS) tablet, phosphoric acid, thiobarbituric acid, 1,1,3,3-tetramethoxypropane, dimethyl sulfoxide (DMSO), SA and 5-FU were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used were of analytical grade. The bicinchoninic acid (BCA) assay kit used for the protein levels of tissue samples determination was purchased from Thermo Scientific (Rockford, IL). Total oxidant status (TOS) and total antioxidant status (TAS) kits were purchased from Rel Assay Diagnostics (Gaziantep, Turkey). Rat catalase (CAT), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Fine Biotech Co. Ltd (Wuhan, China).

Animals

Thirty adult female Sprague-Dawley rats with weighting 150-170 g and aged 7-8 weeks were obtained from the Surgical Practice Research Centre of Karadeniz Technical University. Animals were fed standard rat chow with free access to tap water and maintained under light-darkness for 12 h in a room temperature (22±3°C) and humidity (50±20%) controlled facility. Animals received humane care in accordance with the guidelines of the US National Institutes of Health, and the all stages of experiments were approved by the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol no: 2022/14).

Experimental design

Thirty rats were assigned to 5 groups (n=6/group) designated as: control, 5-FU (100 mg/kg), 5-FU+SA (2.5 mg/kg), 5-FU+SA (5 mg/kg) and SA (5 mg/kg)

groups. 5-FU and SA were administered intraperitoneally (i.p.) to rats. Group 1: Animals in the control group were treated with saline on the first day and DMSO for the following three days. Group 2: Animals in the 5-FU group were administered 5-FU (100 mg/kg) on the first day and DMSO for the following three days. Group 3 and 4: The rats were administered 5-FU (100 mg/kg) on the first day and 2.5 mg/kg and 5 mg/kg of SA for the following three days, respectively. Group 5: Animals in the SA *per se* group were administered saline on the first day and SA (5 mg/kg) for the following three days. 5-FU and SA were prepared by dissolving with saline and DMSO, respectively. The doses of 5-FU[6,14] and SA[15,16] administered to the rats were determined by considering the previous literature. At the end of the fifth days, all rats were sacrificed with cervical dislocation and their ovaries were excised and stored at -80°C for biochemical analysis.[17]

Biochemical analysis

Ovarian tissue was cut into small pieces and homogenized with 2 mL of ice-cold PBS (pH: 7.4) using a homogenizer (IKA, T25 Ultra-Turrax, Staufen, Germany). Residues were removed from the homogenate by centrifugation at 1800xg for 10 min at 4°C. The protein concentrations of the supernatants were determined using a commercial BCA assay kit using bovine serum albumin as the reference standard.

Malondialdehyde (MDA) levels of ovarian tissues were determined by a previously published method.[18] 1,1,3,3-tetramethoxypropane was used as a standard and tissue MDA levels were expressed as nmol/mg protein.

Tissue TOS and TAS levels were determined using commercial colorimetric kits according to the manufacturer's recommendations. The TOS/TAS ratio was used as the oxidative stress index (OSI) and was calculated using the formula[19]:

$$\text{OSI (arbitrary unit)} = \frac{\text{TOS (µmoL hydrogen peroxide equivalent/L)}}{\text{TAS (µmoL trolox equivalent/L)}} \text{ x } 100$$

Tissue CAT, 8-OHdG and IL-6 levels were determined using commercial ELISA kits according to the manufacturer's recommendations. The absorbance measurement of biochemical markers measured by ELISA method was performed using a microplate reader (Molecular Devices Versamax, Sunnyvale, CA, USA) at 450 nm wavelength. CAT, 8-OHdG and IL-6 levels were expressed mIU/mg protein, ng/mg protein and pg/mg protein, respectively.

Statistical analysis

Data were analyzed with Statistical Package for the Social Sciences (Version 23.0, NY, USA). The compliance of the data to normal distribution was evaluated with the Kolmogorov-Smirnov test. Comparisons of the groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical significance was set at $p<0.05$.

RESULTS

The levels of MDA, TOS, OSI, 8-OHdG and IL-6 in the ovarian tissue were significantly enhanced in 5-FU-induced rats compared with normal control rats (112%, p=0.02; 545%, p=0.0001; 1550%, p=0.003; 591%, p=0.0001 and 352%, p=0.001, respectively). SA (2.5 mg/kg) treatment significantly reduced only the levels of 8-OHdG compared with 5-FU-induced rats without treatment (39%, p=0.008). However, SA (5 mg/kg) treatment significantly reduced the levels of MDA, TOS, OSI, 8-OHdG and IL-6 compared with 5-FU-induced rats without treatment (48%, p=0.038; 78%, p=0.002; 92%, p=0.003; 81%, p=0.0001 and 78%, p=0.001, respectively).

The TAS and CAT levels were significantly decreased by 55% (p=0.02) and 49% (p=0.019), respectively in the ovarian tissue of 5-FU-induced rats compared with normal control rats. Treatment with SA (5 mg/kg) significantly increased the reduced levels of TAS and CAT by 55% (p=0.02) and 46% (p=0.038), respectively compared with only 5-FU-treated rats.

In addition, treatment with SA (5 mg/kg) alone did not show any significant change in the any biochemical parameter levels compared with the control group (p>0.05) (Table 1).

Table 1. Effect of SA treatments on ovarian oxidative stress, DNA damage and inflammatory parameters

	Control	5-FU	5-FU+SA (2.5 mg/kg)	5-FU+SA (5 mg/kg)	SA (5 mg/kg)
MDA (nmol/mg protein)	32.7±14.9	69.3±23.8[a]	41.7±25.8	35.9±16.7[b]	29.2±7.6
TOS (μM H_2O_2 equivalent/L)	9.80±0.68	63.2±35.3[a]	36.8±25.0	13.8±6.26[b]	11.1±4.00
TAS (mM trolox equivalent/L)	0.88±0.21	0.40±0.18[a]	0.60±0.33	0.88±0.32[b]	0.82±0.15
OSI (arbitrary unit)	1.17±0.28	19.3±13.7[a]	9.72±9.63	1.62±0.54[b]	1.35±0.55
8-OHdG (ng/mg protein)	17.2±8.0	118.9±22.4[a]	73.0±35.0[a,b]	22.6±12.7[b,c]	20.9±17.1
CAT (mIU/mg protein)	148.8±46.9	76.3±13.0[a]	103.4±18.3	142.3±38.1[b]	158.5±52.9
IL-6 (pg/mg protein)	132.4±46.2	598.9±213.2[a]	347.5±332.7	134.3±32.3[b]	129.4±41.7

5-FU: 5-fluorouracil, SA: sinapic acid, MDA: malondialdehyde, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, CAT: catalase, IL-6: interleukin-6.
P-values according to one-way ANOVA test, post-hoc Tukey test. Data were expressed as mean±SD.
a $p<0.05$ compared with control group,
b $p<0.05$ compared with 5-FU group,
c $p<0.05$ compared with 5-FU+SA (2.5 mg/kg) group.

DISCUSSION

5-FU is one of the most widely used chemotherapeutic drugs in the treatment of various types of cancer. However, tissue toxicity is one of the most important side effects of 5-FU. Because 5-FU acts specifically on tumor cells, but also on normally proliferating cells, which ultimately results in severe toxicity.[20] Tissue toxicity due to 5-FU is thought to be initially caused by increased oxidative stress and associated inflammatory and apoptotic processes.[21] Based on the fact that 5-FU administration causes acute ovarian damage in experimental models in recent years, it was aimed for the first time to evaluate the effects of SA on the ovarian tissue of rats administered 5-FU through oxidative stress and inflammation parameters in the current study.

There is a constant production of ROS in the human body as a result of metabolic processes.[22] In the normal physiological state, ROS production in the body is balanced by the cleansing antioxidant system. This balance is disturbed due to overproduction of ROS and/or insufficiency of the antioxidant system, and this is called oxidative stress.[24] It has been revealed that oxidative stress plays a role in the etiopathogenesis of many pathological conditions, such as aging, cancer, cardiovascular diseases and chemical-induced damage.[10,26] Scientific evidences have shown that oxidative stress plays a central role in mediating 5-FU-induced tissue toxicity. Excessive production of ROS causes membrane lipid peroxidation, carbohydrate, protein and DNA damage and ultimately oxidative cellular destruction.[26] MDA is one of the end products of lipid peroxidation, and an increase in MDA indicates an increase in oxidative stress and a decrease in antioxidant enzymes.[27] It is well known that two of the crucial parameters for evaluating redox balance in biological systems are TAS and TOS. While TAS determines the overall ROS scavenging ability in a biological sample, TOS can be defined as the cumulative amount of total oxidants in the sample. For the quantitative assessment of redox homeostasis disorders, the OSI, which is called the "gold indicator of oxidative stress", is used.[28] Oxidative stress also increases DNA damage, and 8-OHdG is one of the main products of DNA oxidation.[29] Removal of free radicals in biological systems is achieved through enzymatic and non-enzymatic antioxidants, which act as the main defense systems against free radicals.[1,30] CAT, an antioxidant enzyme, prevents oxidative stress-induced cell damage by catalyzing the breakdown reaction of H_2O_2 to water and oxygen.[29] The role of oxidative stress and oxidative stress-induced DNA damage in 5-FU-induced ovarian toxicity was evaluated by analyzing markers of MDA, TOS, TAS, OSI and 8-OHdG in this study. In the current study, ovarian tissue of rats treated with 5-FU exhibited higher levels of MDA, TOS, OSI and 8-OHdG, but lower levels of TAS and CAT, compared to normal rats. These findings are consistent with data from previous studies showing that 5-FU increases oxidative stress and DNA damage and suppresses the antioxidant system.[2,6,20,26,31] SA treatments (2.5 and 5 mg/kg) significantly and dose-dependently attenuated oxidative stress, as demonstrated by decreased lipid peroxidation, DNA damage and renewed antioxidant levels compared to 5-FU-treated rats alone. SA is considered an important chain-breaking antioxidant that functions efficiently as

a radical scavenger. The role of SA as an antioxidant is related to its hydrogen atom donating ability and its ability to stabilize phenoxyl radicals generated through the conjugate system. It is also reported that the antioxidant function of SA is much more important than ferulic acid and caffeic acid.[8] Alleviation of oxidative stress and DNA damage parameters by SA treatments suggested that SA is due to its free radical scavenging potential and hydrogen atom donating ability. These results confirmed the results of previous reports.[11,27,32,33]

Oxidative stress and inflammatory processes are closely related, and inflammatory cytokines play an important role in 5-FU-induced acute tissue injury.[21] IL-6 is a very important cytokine involved in the pro-inflammatory process and there is a positive correlation between increased IL-6 levels and the degree of inflammation.[6] The role of inflammation in 5-FU-induced ovarian toxicity was therefore evaluated by analyzing marker of IL-6 in this study. Ovarian tissue of rats treated with 5-FU exhibited higher levels of IL-6 compared to normal rats. These findings were consistent with data from previous studies showing that 5-FU increases tissue inflammation levels.[20,21,26,34] However, IL-6 levels were found to be significantly reduced in the ovarian tissue of rats which received SA treatment for 3 days after 5-FU injection. This alleviation may be due to the effective anti-inflammatory activity of SA resulting from its ability to regenerate the antioxidant system.[33] Consistent with our results, previous studies have reported that SA can exert beneficial effects in various models of chemical-induced organ damage, through its ability to modulate pro-inflammatory cytokines, including IL-6, tumor necrosis factor-alpha, interleukin-1 beta, myeloperoxidase and nuclear factor-kappa B.[12,13,27,35,36]

CONCLUSION

We demonstrated using an *in vivo* model that SA can exert an ovoprotective effect against 5-FU-induced ovarian injury for the first time. This is most likely due to the antioxidant and anti-inflammatory activity of SA. The use of natural antioxidants as potential therapeutic agents in the abolition of chemotherapeutic-induced tissue damage is of increasing interest. Our data suggest that SA may also be useful in ameliorating 5-FU-induced ovarian injury. However, more extensive preclinical studies are required to prove its clinical efficacy.

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Authorship contribution statement

Consept and desing: SD.

Acquisition of data: SD, AL, EAD and NTA.

Analysis and interpretation of data: SD, AM, EAD and YA.

Drafting of the manuscript: SD.

Critical revision of the manuscript for important intellectual content: YA.

Statistical analysis: AM.

Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

Ethical approval

This study was approved by the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol no: 2022/14) and performed according to the animal research reporting of *in vivo* experiments (ARRIVE) guidelines.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Yağ Dokusunda Katalaz Aktivitesi Ölçümü için Farklı İzolasyon Yöntemlerinin Karşılaştırılması

Comparison of Different Isolation Methods for Measuring Catalase Activity in Adipose Tissue

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ÖZET

Son zamanlarda yapılan çalışmalar yağ dokusunun yalnızca enerji deposu olmadığını, bunun yanında endokrin bir organ olduğunu da gösterdi. Vücutta artan yağ kitlesi sonucu oluşan obezite artmış oksidatif stres ve düşük dereceli kronik inflamasyonla birlikte gözlenmektedir. Yağ dokusunda gözlenen inflamasyona bağlı olarak dokuda oksidatif stres de artmaktadır. Antioksidan enzimler, daha az aktif radikal oluşmasına yol açarak veya serbest radikal zincir reaksiyonunun proteinler, lipidler, karbohidratlar ve DNA üzerine hasarını azaltarak oksidatif stresin şiddetini bastırmaya yardımcı olan proteinlerdir. Önemli bir antioksidan enzim olan katalaz (CAT), H_2O_2'yi su ve oksijene parçalayarak oksidatif stresin oluşumunu engeller. Yağ dokusunun yüksek lipit ve düşük protein içeriğine sahip olması, lipit interferansının yüksek olması bu dokuda protein izolasyonunu ve aktivite ölçümlerini zorlaştırmaktadır. Çalışmamızda, yağ dokusunda farklı protein izolasyon yöntemlerinin kullanılmasının CAT aktivitesi üzerine etkisinin incelenmesi ve ölçüm için gerekli şartların ortaya konulması amaçlandı. CAT aktivitesi ölçümleri Aebi yöntemi kullanılarak yapıldı. Sıçanlardan retroperitoneal yağ dokusu çıkarılıp üç farklı homojenizasyon yöntemi kullanılarak aktivite ölçümleri gerçekleştirildi. Homojenizasyon 1 (H1)'de organik çözücü olarak kloroform/metanol, homojenizasyon 2 (H2)'de ve homojenizasyon 3 (H3)'te sadece kloroform kullanıldı. Yapılan değerlendirmeler sonucunda H1 yönteminin ortalama spesifik aktivite değerleri diğer iki yönteme göre daha yüksek bulundu. Sonuç olarak, retroperitoneal yağ dokusunda H1 yönteminin CAT enzim aktivitesi ölçümünde daha uygun olabileceği kanaatine varıldı.

Anahtar Kelimeler: Adipoz Doku, Enzim Aktivitesi, Katalaz

ABSTRACT

Recent studies have shown that adipose tissue is not only an energy store, but also an endocrine organ. Obesity, which occurs as a result of increased fat mass in the body, is observed together with increased oxidative stress and low-grade chronic inflammation. It is an increase in tissue oxidative stress due to the inflammation observed in adipose tissue. Antioxidant enzymes are proteins that help suppress the severity of oxidative stress by leading to the formation of fewer active radicals or reducing the damage of a free radical chain reaction on proteins, lipids, carbohydrates, and DNA. Catalase (CAT), an important antioxidant enzyme, prevents the formation of oxidative stress by breaking down H_2O_2 into water and oxygen. The fact that adipose tissue has a high lipid and low protein content, high lipid interference, makes protein isolation and activity measurements in this tissue difficult. In our study, it was aimed to investigate the effect of using different protein isolation methods on CAT activity in adipose tissue and to identify the necessary conditions for measurement. CAT activity measurements were performed using the Aebi method. Retroperitoneal adipose tissue was removed from the rats and activity measurements were performed using three different homogenization methods. Chloroform/methanol was used as the organic solvent in homogenization 1 (H1), only chloroform was used in homogenization 2 (H2) and homogenization 3 (H3). As a result of the evaluations, the average specific activity values of the H1 method were found to be higher than those of the other two methods. As a result, it was concluded that the H1 method may be more suitable for measuring CAT enzyme activity in retroperitoneal adipose tissue.

Keywords: Adipose Tissue, Catalase, Enzyme Activity

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GİRİŞ

Obezite, dünyada ve ülkemizde gün geçtikçe artan önemli bir toplum sağlığı sorunudur ve kısaca fazla enerji alınımına bağlı yağ dokusu artışı olarak tanımlanır. Yağ dokusu vücutta enerjinin depolandığı ana doku olmasının yanı sıra, endokrin bir organ olarak birçok fizyolojik ve patolojik olayda rol alan adipokinleri de salgılamaktadır.[1,2] Salgılanan adipokinler obezite, metabolik sendrom, tip 2 diyabet, hipertansiyon ve kanser gibi birçok hastalığın patolojisinde önemli rol oynamaktadır.[3] Bahsedilen hastalıkların moleküler ve biyokimyasal mekanizmalarının ortaya çıkarılmasında yağ dokusunda bulunan protein karakterli moleküllerin izolasyonu ve ölçümleri oldukça önemli ve bilgi vericidir.

Vücutta veya bir dokuda oksidan-antioksidan dengesinin oksidanlar lehine kayması ile oksidatif stres oluşur. Oldukça farklı karakterde oksidan bileşik bulunmasına rağmen, organizmada oksidatif stresten genellikle reaktif oksijen türler sorumludur. Reaktif oksijen türleri aerobik hücresel metabolik süreçlerde sürekli olarak üretilir. Süperoksit ve hidrojen peroksit gibi serbest radikaller lipitler, proteinler ve DNA gibi çeşitli hücre içi moleküllerle reaksiyona girerek hücrenin yapı ve fonksiyonlarına zarar verir. Obezite, tip 2 diyabet, kalp-damar hastalıkları ve kanser gibi hastalıklarda artmış oksidatif stres patolojide önemli rol oynar. Obezitede hipertrofi ve hiperplazi nedeniyle büyüyen yağ dokusu, adiposit disfonksiyonuna bağlı olarak, normal adipokin profilinin dışına çıkarak inflamatuar süreci tetikleyen bir adipokin salgı profili kazanır (yüksek leptin, TNF-α, IL-6 ve düşük adiponektin salgısı gibi). Özellikle bu dokudan salgılanan inflamatuar sitokinlerin artışı bölgede bulunan makrofaj ve monositlerden reaktif oksijen ve azot türlerinin sentezinin artmasına yol açar. Ayrıca, artan mitokondriyal oksidasyon ve mekanik yükte yağ dokusunda oksidatif stresi daha da derinleştirir. Yukarıda bahsedilen etkiler sonucu obezite düşük seviyeli kronik inflamatuar bir süreç olarak tanımlanır.[3,4]

Oksidatif stres aynı zamanda yetersiz antioksidan savunma mekanizmalari ile de ilgilidir. ROT hücre içi konsantrasyonu, bu bileşiklerin üretimi ve bunların antioksidanlarca ortamdan uzaklaştırılma hızlarına bağlıdır. Hücreler oksidanların neden olacağı hasarı engellemek ya da tamir etmek için birçok antioksidan mekanizmaya sahiptir. Özellikle antioksidan enzimlerden, Süperoksit dismutaz (SOD), katalaz (CAT) ve glutatyon peroksidaz (GPX) oksijen kullanan tüm hücrelerin canlılığı için gereklidir. Bu enzimlerin sıralı reaksiyonları sonucu süperoksit radikali suya indirgenmektedir.[5]

Katalaz (E.C. 1.11.1.6) (CAT) dört alt birimden meydana gelen antioksidan enzimdir. Her bir alt birim, NADPH molekülü ve hem grubu ihtiva eder. CAT enzimi hidrojen peroksitin, O_2 ve H_2O'ya dönüşümünü katalizler. Hidrojen peroksit bir radikal kaynağı olduğu halde, biyolojik önemi olan moleküllerin çoğu ile reaksiyona girmez. Cu^{2+} ve Fe^{2+} iyonlarının katalizörlüğünde Fenton reaksiyonu ile en reaktif oksijen türü olan hidroksil radikali oluşumunda bir ön madde olarak rol oynamaktadır.[6]

Obeziteye bağlı oksidatif stresin değerlendirilmesinde de yağ dokusunda bu enzimlerin ölçümü yapılmaktadır.[7] Ayrıca obezite fizyopatolojisi ile ilgili moleküler çalışmalarda ve tedaviye yönelik uygulamaların değerlendirilmesi kullanılan, proteomik, ELISA, western blot ve enzim ölçümleri gibi yöntemler için yağ dokusundan protein izolasyonu gerekmektedir. Beyaz yağ dokusu, yüksek yağ içeriğine sahip olması ve protein miktarının toplam doku kütlesinin %2'sinden daha az olması sebebiyle proteinlerinin izolasyonu ve analizi yönünden oldukça problemli bir dokudur. Ayrıca lipit interferansının yüksek olması analitik yöntemlerin uygulanmasını güçleştirmektedir. Bu çalışmada, retroperitoneal yağ dokusunda CAT aktivite tayininde kullanılabilecek literatürlerde geçen üç farklı homojenizasyon yöntemi karşılaştırılıp, CAT aktivitesi için en iyi spesifik aktiviteyi gösteren homojenizasyon yönteminin belirlenmesi ve ölçüm için gerekli şartların ortaya konulması amaçlandı.

METOD

Beyaz yağ dokusu örnekleri Recep Tayyip Erdoğan Üniversitesi Deney Hayvanları Araştırma ve Uygulama Merkezi'nde farklı çalışmalar için sakrifiye edilmiş erkek *Sprague Dawley* ırkı ratların retroperitoneal yağ dokuları alınarak temin edildi. Alınan yağ dokusu örneklerine üç farklı lipit homojenizasyon yöntemi uygulandı.

Homojenizasyon Yöntemi 1

İzolasyon Tamponu Hazırlanması (pH 7.4)

0.606 g Tris, 0.87 g NaCl ve 0.08 g EDTA tartıldı. pH 7.4'e ayarlandı ve son hacim 100 mL'ye tamamlandı. Yaklaşık 100 mg yağ dokusu cam tüplere alındı. 200 µL izolasyon tamponu ve 750 µL kloroform/metanol karışımı (2:1) ilave edilerek homojenize edildi.

Homojenatlar ependorflara aktarılarak 20 dk buzda bekletildikten sonra 250 µL kloroform ve 250 µL saf su eklendi ve ependorflar vortekslendi. +4^{0}C 15000 rpm'de 10 dk santrifüj edildi. Santrifüj sonrasında iki faz gözlendi. Üstteki süpernatan temiz ependorfa aktarılarak CAT ölçümü için hazır hale getirildi.[7]

Homojenizasyon Yöntemi 2

HEPES Tamponunun Hazırlanması (25 mM, pH 8.80)

1.63 g HEPES tartıldı. pH 8.80'e ayarlandı ve son hacmi 250 mL'ye tamamlandı. Bir önceki homojenizasyon yöntemi modifiye edilerek işlem gerçekleştirildi. Yaklaşık 100 mg yağ dokusu cam tüplere alındı. 200 µL 25 mM HEPES tamponu çözeltisi içinde homojenize edildi. Homojenatlar ependorflara aktarıldı ve 200 µL kloroform ilave edilerek +4^{0}C'de 15000 rpm' de 10 dk santrifüj edildi. Santrifüj ardından iki faz gözlendi. Üstteki süpernatan fazı temiz ependorfa aktarılarak CAT ölçümü için hazır hale getirildi.[8]

Homojenizasyon Yöntemi 3

Homojenizasyon Tamponu (pH 7.4)

Bir balon jojenin içine sıvı triton X-100 50 µL ilave edildi ve üzerine 100 mL saf su ilave edilerek çözelti hazırlandı. 0.05 M 100 mL Tris-HCl hazırlamak için bu maddeden 0.788 gr tartıldı ve bir miktar saf suda çözünmesi sağlandıktan sonra 100 mL' ye tamamlandı. Hazırlanan iki çözelti birbirine karıştırıldıktan sonra pH 7.4' e ayarlanarak homojenizasyon tamponu hazırlandı. 150 mg yağ dokusu tartılarak buz içine alındı. Buz üzerinde bekleyen dokunun üstüne 2 mL soğuk homojenizasyon tamponu ilave edildi. Dokular soğuk ortamda 10 saniye boyunca homojenizatörle homojenize edildi. Homojenize doku +4 °C'de, 3000 rpm' de, 10 dakika santrifüj edildi. En üsteki yağ tabakası bir pastör pipet yardımıyla uzaklaştırılarak süpernatan alındı. 1 mL süpernatan üzerine 300 µL kloroform konuldu. Karışım vortekslenerek +4^{0}C' de, 10.000xg'de 15 dk santrifüj yapıldı. Elde edilen süpernatan CAT ölçümü için hazır hale getirildi.[9]

CAT aktivitesi Aebi yöntemi ile ölçüldü.[10] Tablo 1'de sunulan şekilde pipetlemeler yapılarak 240 nm'de H_2O_2'nin bozulması sonucu absorbanstaki düşüş takip edildi. Katalaz aktivitesinde birim olarak birinci derece reaksiyon hız sabiti (k) kullanıldı.[10] Dört faklı dilüsyon seviyesinde ikili tekrarlar halinde ölçümler yapıldı. Retroperitoneal yağ dokularından elde edilen homojenatlar da protein tayini için bisinkoninik asit (BCA) protein ölçüm kiti (Thermo Scientific, Pierce™ BCA Protein Assay Kit) kullanıldı.

Tablo 1. Katalaz aktivitesi ölçümü için reaksiyon karışımı

Reaktifler	Kör (mL)	Numune (mL)
Fosfat tamponu (50 mM, pH 7.0)	0.25	-
Numune	0.50	0.50
H_2O_2 (% 30)	-	0.25

İstatiksel Analiz

Elde edilen sonuçlar ortalama olarak ifade edildi. Karşılaştırmalar Kruskal Wallis testi ile yapıldı ve post hoc olarak ta Mann Whitney U testi kullanıldı. $p< 0.05$ istatiksel olarak anlamlı kabul edildi.

SONUÇLAR

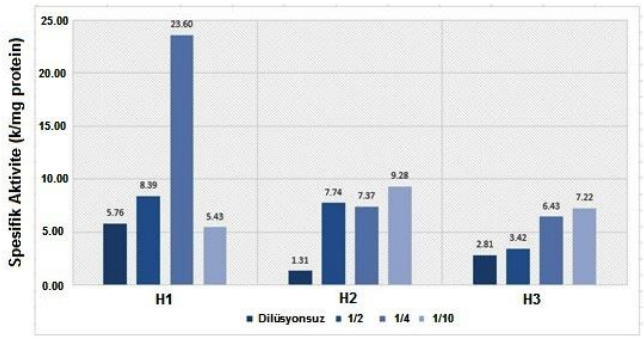
Homojenizasyon yöntemlerinde ölçülen CAT aktivitelerini spesifik aktiviteye çevirmek için kullanılan protein miktarları farklı dilüsyonlar için Tablo 2'de verilmiştir. Yapılan istatiksel değerlendirmeler sonucunda H1 ile H2 yönteminden elde edilen ortalama total protein miktarları arasında fark gözlenmemiştir ($p=0.059$). H3 yönteminde diğer iki yönteme göre anlamlı seviyede daha fazla protein miktarı elde edilmiştir ($p<0.05$).

Her üç homojenizasyon yönteminin de farklı dilüsyonlardaki spesifik aktiviteleri Şekil 1'de gösterilmiştir. Yapılan istatiksel değerlendirmeler sonucunda üç yöntem arasında istatiksel olarak anlamlı bir fark gözlenmemiştir ($p=0.886$).

Tablo 2. Kullanılan 3 yöntemde elde edilen protein miktarı sonuçları (mg/mL)

Seyreltme oranı	H1	H2	H3
Dilüsyonsuz	0.274	0.646	0.994*
1/2	0.176	0.242	0.522*
1/4	0.122	0.117	0.354*
1/10	0.083	0.044	0.177*

*H1 ve H2'ye göre istatistiksel olarak anlamlı farklı ($p<0.05$).



Şekil 1. Üç homojenizasyon yönteminin spesifik aktiviteleri

TARTIŞMA

Bu çalışmasında, yağ dokusu örneklerinde CAT enzim aktivitesi ölçümünde kullanılabilecek organik solventle doku homojenetların delipidasyonu (lipidlerin uzaklaştırılması) esasına dayanan üç farklı yöntem karşılaştırılmıştır. Yağ hücreleri yapısal olarak sitoplazmalarında triaçilgliserol ve az miktarda kolesterol ve fosfolipit içeren lipit damlacıklarını içerir. Sitoplazmadaki lipit damlacıkları perilipin denilen bir proteince sarılmıştır. Bu hidrofobik proteinlerin klasik deterjan kullanan yöntemlerle izolasyonu zordur. Proteinler oluşan misel yapılarının içinde kalmakta ve izole edilen protein miktarı azalmaktadır. Ayrıca, deterjanların birçok ölçüm için interferans yapmaktadır.[7]

Kullanılan homojenizasyon yöntemlerin etkinliği ortaya koymak için spesifik aktivite değerleri hesaplanmış ve değerlendirilmiştir. Kullanılan ilk homojenizasyon yönteminde (H1) yağ dokusu Sajic ve ark.[7] tarafından tanımlanan yöntem modifiye edilerek homojenize edilmiştir. İzolasyon tamponu ile yapılan homojenizasyonu takiben kloroform/metanol (1:2) karşımı ile delipidasyon işlemi gerçekleştirilmiştir. İkinci homojenizasyon yönteminde (H2) HEPES tamponu ile yapılan homojenizasyonu takiben kloroform ile delipidasyon işlemi yapılmıştır. Üçüncü homojenizasyon yönteminde (H3) yağ dokusu homojenizasyonu yine homojenizasyon ve deterjan (triton X-100) ile yapılmış, delipidasyon işlemi sadece kloroform kullanılarak gerçekleştirilmiştir. Elde edilen sonuçlar (Tablo 1) incelendiğinde en yüksek protein değerlerinin H3'te olduğu gözükmektedir. H3'te en yüksek protein içeriğinin olmasında polar organik çözelti kullanılmamasının bir sonucu olabilir. Proteinler organik çözücülerle muamele edildiklerinde çökerler. Bununla beraber, bazı hidrofobik proteinler, özellikle hücre zarlarında yerleşenler organik çözücüler etkisi ile çöktürülemezler. Hücre zarlarında kullanılan organik çözücüler proteinin hidrofobik parçaları etrafındaki su moleküllerinin yerini alırlar. Buda, protein çözünürlüğün artması ile sonuçlanır. Ayrıca bu homojenizasyon yönteminde kullanılana deterjanda protein çözünürlüğünü arttırmış olabilir. Proteinlerin molekül büyüklüğüne bağlı olarak da çökme davranışını değiştirmektedir. Büyük molekül ağırlıklı proteinler diğer özellikleri aynı olan daha küçük molekül ağırlıklı proteinlere göre daha düşük organik çözücü konsantrasyonların da çökerler.[11] Homojenizasyon yöntemleri içersinde tampon/klorofom oranı en yüksek homojenizasyon yöntemi H3'tür. Bu sebebe bağlı olarak H3'te ölçülen protein miktarı daha yüksek olabilir.

CAT hücrelerde SOD tarafından üretilen hidrojen peroksiti su ve oksijene dönüştüren enzimdir. CAT'ın çoğunluğu peroksizomlarda lokalize olmuştur. İnflamatuvar bir süreç olan obezitede, NADPH oksidazlar oksidatif stresi indükleyebilir. Bu enzim bol miktarda süperoksit anyonu oluşturmaktadır. Süperoksidin zararlı etkilerinin bertaraf edilmesinde ilk savunma hattı SOD aktivitesidir. Bu aktiviteye bağlı oluşan H_2O_2 de CAT ve glutatyon peroksidaz aktiviteleri ile bertaraf edilir.[5]

Farklı seyreltme oranlarında üç homojenizasyon yöntemi ile elde edilen spesifik aktivite değerleri Şekil 1'de verilmiştir. En yüksek spesifik aktivite değeri H1'de elde edilmiştir, ancak istatistiksel olarak bu fark anlamlı bulunmamıştır ($p=0.886$). H1 yöntemi en düşük ortalama protein değerlerine sahip olmasına rağmen en yüksek CAT aktivitesine sahiptir. Her üç homojenizasyon yönteminde de yukarıda anlatıldığı gibi apolar bir organik çözücü (kloroform) ve H1 de polar organik çözücü (metanol) kullanılmıştır. Genel olarak kullanılan organik çözücüler izole edilecek proteinlerin üç boyutlu yapılarını değiştirerek çözünürlüklerinin kaybetmelerine ve çökmelerine sebep olur. Bahsedilen etki 0ºC üzerindeki şartlarda daha belirgindir. Bu yüzden denatürasyon çalışmalarında, proteinleri çöktürmek için organik çözücüler sıklıkla kullanılır. Organik çözücüler biyolojik örneklerde tüm proteinlerin çökmesini sağlayabileceği gibi, uygun deneysel şartlarda bazı protein fraksiyonlarının çökmeden fonksiyonel olarak ortamda kalmasını sağlayabilir. Bu yöntem (polar organik çözücü kullanımı) enzim izolasyon çalışmalarında sıklıkla kullanılmaktadır.[12] Enzim katalizi ile ilgili çalışmalarında, substrat (lar) organik çözücülerde daha fazla çözünürlüğe sahip olduğu, arzulanan yönlerde reaksiyon dengelerinin değiştirilmesi, kontaminasyon riskinin azaltılması, termostabilitenin artırılması, enzimin geri kazanımı ve tekrar kullanılabilirliği, uçucu çözücüler kullanıldığında enerji verimliliğinin artırılması, asit anhidritler gibi "neme duyarlı" substratlar/reaktifler kullanması gerektiğinde, substratın özgüllüğü, özgünlüğüne ve enantioselektiflik gibi durumlarda organik çözücü kullanımı tercih edilir.[13] Kullanılan organik çözücü, enzimlerin tersiyer ve kuaterner yapılarını etkileyerek ölçümler sırasında

domainlerin esnekliğini, subsratların aktif bölgeye ulaşması ve bağlanması gibi özellikleri değiştirerek enzim aktivitesi üzerine olan etkisi gerçekleştirebilir.[14] Polar organik çözücülerden metanol ve etanol gibi alkollerin, gliseraldehid 3-fosfat dehidrogenaz, fruktoz 1,6-bisfosfataz ve alkalen fosfataz gibi enzimlerin aktivitesini önemli ölçüde arttırdığı gösterilmiştir [14]. Bu olayın kesin nedeni bilinmemekle birlikte, alkollerin peptitlerde ve proteinlerde yapısal değişiklikler meydana getirdiği uzun zamandır bilinmektedir. Alkoller enzimlerin konformasyonel esnekliğini artırarak katalitik aktivite için moleküler bir kayganlaştırıcı gibi davranmaktadır.[14]

H1'de yağ dokusu homojenize edildikten sonra kullanılan klroroform/metanol karşımı H2'de kullanılan kloroforma göre CAT aktivitesini daha iyi korumuştur. Bunda mevcut aktivitenin korunması kadar metanolün CAT aktivitesini yukarıda bahsedilen şekilde artırmış olmasının bir sonucu olabilir. H1 ve H2'de hemen hemen aynı miktarda protein içeriği bulunmasına rağmen CAT aktivitesinin H2'de daha düşük olmasının nedeni, proteinin denatürasyonla aktivitesini kaybetmesi ve /veya kullanılan klorofom miktarının CAT fraksiyonunu çözünür halde tutamamasının sonucu olabilir. Ayrıca, organik çözücüler su ile karıştırıldığında ısı üretimine sebep olmaktadır ve bu denatürasyonu daha da artırmaktadır.[12] H1'de buz üzerinde numuneler bekletilirken H2'de bu uygulama yapılmamıştır. H2 için soğuk ortamın sağlanmamış olması denatürasyonu artırmış olabilir. Yukarıda bahsedildiği gibi organik çözücüler subsratların çözünürlüğü üzerinde de etkilidir. Metanolün kloroforma göre daha polar olması deneylerde kullanılan H_2O_2'nin çözünürlüğü üzerinden de enzim aktivitesinin H1' de daha yüksek olmasına sebep olmuş olabilir. Elde ettiğimiz sonuçlar Aebi yöntemi ile CAT aktivitesi ölçümü ile ilgilidir. CAT aktivitesi ölçümünde kullanılacak farklı yöntemlerde (Goth yöntemi gibi) substrat değişikliği gibi faktörler farklı sonuçların elde edilmesine sebep olabilir. H3'de en yüksek protein miktarına rağmen en düşük CAT aktivitesi ölçülmesinde deterjan kullanılması etkili olabilir. Triton X-100'ün deterjan etkisi lipid-protein ayrımını tam anlamıyla yerine getirirken, yüksek miktarda ürün elde edilmesine imkan sağlarken, apolar karakterinden dolayı proteinlerde ciddi denatürasyona yol açmış olabilir. Yine yüksek apolariteden dolayı subsrat çözünürlüğünün azalması, elektron transferinin zorlaşması gibi olaylar CAT'ın aktivite kaybına uğramasına sebep olmuş olabilir. Ayrıca H2 yöntemindeki kullanılan pH' nın 8.8 değerine sahip olması da katalaz aktivitesini değiştirmiş ve daha düşük spesifik aktiviteye sebep olmuş olabilir.

SONUÇ

Sonuç olarak, retroperitoneal yağ dokusunda Aebi yöntemi ile CAT aktivite tayininde kullanılabilecek üç farklı homojenizasyon yöntemi karşılaştırıldığı çalışmamızda, H1 yönteminin (kloroform /metanol karışımı) CAT aktivitesi tayininde daha yüksek spesifik aktiviteye sahip olduğu gözlendi ve bu etkide daha polar özelliğe sahip metanol varlığının ve soğuk ortam uygulamasının önemli olabileceği kanaatine varıldı.

Yazarlık katkı beyanı

Konsept ve dizayn: AA, SU
Verilerin eldesinde: AA, SU
Verilerin analizinde ve yorumlanmasında: AA, SU
Makale yazımında: AA, EŞ
Makale revizyonu ve entelektüel katkı: AA, EŞ
Gözetiminde: AA

Yazar çıkar çatışması

Yazarların arasında potansiyel çıkar çatışması yoktur.

Destek

Bu çalışma için hiç bir maddi destek alınmamıştır.

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Cloning, Recombinant Production and Functional Analysis of Staphylococcal Phage Endolysins

Stafilokkal Faj Endolizinlerinin Klonlanması, Rekombinant Olarak Üretilmesi ve Fonksiyonel Analizleri

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ABSTRACT

Staphylococci are pathogens that cause serious infections in humans and animals. Nosocomial infections caused by staphylococci, particularly methicillin-resistant *Staphylococcus aureus* (MRSA) strains, are mostly transmitted through healthcare workers, patients, or contaminated materials and food. In recent years, studies have been carried out to develop alternative antimicrobial strategies due to the inadequacy of existing antibiotics in the prevention of systemic, skin and implant-related biofilm infections caused by these multi-antibiotic resistant strains. One of these new approaches is the development of products containing the bacteriophage endolysin, which is particularly effective against multi-antibiotic-resistant bacteria. In this study, endolysin genes of bacteriophages (prophages) integrated into the chromosomes of *Staphylococcus* strains were amplified by polymerase chain reaction (PCR) and cloned into pET SUMO and pET-30b(+) vectors and produced recombinantly in *E. coli*. Anti-staphylococcal and antibiofilm activity of recombinant endolysins against *S. aureus*, *S. epidermidis*, and *S. haemolyticus* strains isolated from clinical specimens, were demonstrated using turbidity reduction, biofilm removal in microwell plates by crystal violet method, and capacity of endolysins to kill biofilm-forming bacteria by confocal microscopy imaging by live-dead staining. The combination of endolysin was shown to reduce bacterial culture turbidity by at least 50% at 60 minutes and biofilms by approximately 70% at 12 hours. These results show that endolysins have the potential to be used in the prevention of staphylococcal infections.

Keywords: *Staphylococcus*, Bacteriophage, Anti-biofilm, Endolysin, Confocal Microscopy

ÖZET

Stafilokoklar insanlarda ve hayvanlarda ciddi enfeksiyonlara neden olan patojenlerdir. Stafilokokların, özellikle metisiline dirençli *Staphylococcus aureus* (MRSA) suşlarının neden olduğu nozokomiyal enfeksiyonlar çoğunlukla sağlık çalışanları, hastalar veya kontamine olmuş maddeler ve yiyecekler yoluyla bulaşır. Son yıllarda bu çoklu antibiyotik dirençli suşların neden olduğu sistemik, deri ve implant ilişkili biyofilm enfeksiyonlarının önlenmesinde mevcut antibiyotiklerin yetersizliği nedeniyle alternatif antimikrobiyal stratejiler geliştirmeye yönelik çalışmalar yapılmaktadır. Bu yeni yaklaşımlardan biri, çoklu antibiyotiğe dirençli bakterilere karşı özellikle etkili olan bakteriyofaj endolizin enzimini içeren ürünlerin geliştirilmesidir. Bu çalışmada, *Staphylococcus* suşlarının kromozomlarına entegre olmuş, bakteriyofajların (profaj) endolizin genleri polimeraz zincir reaksiyonu (PCR) ile çoğaltılarak pET SUMO ve pET-30b(+) vektörlerine klonlandı ve *E. coli*'de rekombinant olarak üretildi. Rekombinant endolizinlerin, klinik örneklerden izole edilen *S. aureus*, *S. epidermidis* ve *S. haemolyticus* suşlarına karşı anti-stafilokokal ve antibiyofilm etkileri bulanıklılık azaltma, mikrokuyucuklu plaklarda kristal viyole yöntemi ile biyofilm uzaklaştırma ve konfokal mikroskopi yöntemi ile endolizinlerin biyofilmi oluşturan bakterileri öldürme kapasiteleri canlı ve ölü bakteri görüntüleme yöntemi ile saptandı. Endolizin kombinasyonunun bakteri kültür bulanıklılığını 60 dakikada en az %50 ve biyofilmleri 12 saatte yaklaşık %70 oranında azalttığı gösterildi. Bu sonuçlar endolizin enzimlerinin stafilokokal enfeksiyonların önlenmesinde kullanılma potansiyeline sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Staphylococcus*, Bakteriyofaj, Biyofilm, Endolizin, Konfokal Mikroskopi.

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INTRODUCTION

The genus *Staphylococcus* includes more than 30 different species responsible for various infections. Most of these infections are caused by *Staphylococcus aureus* (*S. aureus*), and many are found in the human microbiota.[1] Nosocomial infections often associated with S. *aureus*, including methicillin-resistant *S. aureus* (MRSA), is transmitted primarily through contaminated medical devices or direct contact with colonized healthcare workers or patients. *S. aureus*, *S. epidermidis*, and other coagulase-negative staphylococci are responsible for 30% of nosocomial infections.[2] They cause complications, especially in people with suppressed immune systems. They can lead to a prolonged hospital stay and even death due to other complications. Therefore, alternative approaches are needed to treat these infections. One such approach involves the use of lytic bacteriophages alone or in combination with conventional antimicrobial agents.[3] There has been increased interest in studies to investigate the antibiotic or enzyme-based antibacterial potentials of *Streptococcus pneumoniae*, [4] *S. aureus*,[5, 6] *Enterococcus faecalis*,[7] *Bacillus anthracis*,[8] and *Pseudomonas aeruginosa*,[9] phages. There many studies about the prevention of the formation of biofilm or removing the biofilm structure by the endolysin enzymes. A broad-spectrum phage endolysin (LysCP28) showed both antibacterial, biofilm removal and biofilm formation inhibition activity against *Clostridium perfringens*.[10] Simalarly, *S. aureus*,[11,12] *Streptococcus suis*,[13] *Streptococcus pyogenes*,[14] *Gardnerella vaginalis*[15] biofilms are eliminated by endolysins. Endolysins are enzymes encoded and released by phages in the final stage of their life cycle to release newly produced phage particles by disrupting the host bacterial cell wall.[16] When recombinant endolysin is added exogenously in a culture of gram-positive bacteria, unlike regular life cycles, the bacteria result in a short breakdown without the aid of holin proteins.[6,17,18] These properties make them attractive antibacterial agents. There are several advantages to using phage endolysins over phages and conventional antimicrobial agents because they can kill susceptible bacteria with which they come into contact. Since phage endolysins inactivate target bacteria specifically with a lytic effect without damaging the normal microflora, it is very rare for bacteria to develop resistance against them.[19] These properties of phage endolysins have enabled them to be used in various fields, such as medicine, veterinary medicine, agriculture, the food industry, and biotechnology.[16,20] In another study, an endolysin enzyme was produced recombinantly and called PlyC, was used as a disinfectant on inanimate solid surfaces and showed a disinfection effect by breaking down the peptidoglycan layer of the bacteria and showed an antibacterial effect against many Streptococcus species, including *Streptococcus equi*. They report it to be 1000 times more effective when compared to commercial disinfectants. It was determined that 1 mg of PlyC showed an antibacterial effect within 30 minutes against *S. equi* cells at a concentration of 10^8 CFU/mL. Based on these findings, the researchers put PlyC through a standardized series of tests, including the dilution methods used for disinfectants and germicide sprays prescribed by the Association of Official Analytical Chemists (AOAC).[21] In this present study, six endolysin genes belonging to bacteriophages that lysogenize *S. aureus* strains isolated from food and food workers were amplified by PCR and cloned into pET SUMO and pET-30b(+) expression vectors and recombinantly produced in *E. coli*. A mixture containing recombinant endolysins was observed to be anti-staphylococcal and antibiofilm effective against *S. aureus*, *S. epidermidis*, and *S. haemolyticus* strains isolated from clinical specimens.

METHODS

Bacterial Strains and Culture Conditions

A total of 173 *S. aureus* strains obtained from the Samsun Veterinary Control Institute were included in this study. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) media were used for culturing *S. aureus* strains and phage endolysin activity tests. The broth and agar media were prepared following the manufacturer's instructions, and sterilized by autoclaving before use. Both broth and agar cultures were incubated under aerobic conditions at 37°C, and the broth cultures were incubated by shaking at 220 rpm. The *E.coli* strains were routinely cultured in Luria-Bertani (LB) broth and agar (ThermoFisher Scientific, USA).

Phage Endolysin Gene Amplification

The chromosomal DNA from *S. aureus* strains was isolated by the standard lysostaphin-phenol/chloroform method using 3 mL overnight broth cultures. Following the chromosomal DNA isolation, the presence of the endolysin gene was screened by PCR. The pET SUMO (Invitrogen, USA) and pET-30b(+) vectors (Merck, Germany). The following two sets of primers designed

based on the published endolysin genes of *S. aureus* DW2 phage (Acc. No. KJ140076) stored in the gene bank were used: Lys-F1: 5'-ATG CGT AGA ATA AGA AGA CCT AAG -3' and Lys-R1: 5'-TTA TTT CTT ATC GTA AAT GAA TTG -3' for pET SUMO cloning and expression; ELys-F: 5'- TAC TTA CAT ATG ATG CAA GCA AAA CTA ACT AAA AAA G-3' with *NdeI* restriction site, and ELys-R: 5'-GTG CTC GAG TTA ACT GAT TTC TCC CCA TAA GTC-3' with *XhoI* restriction site, and cloned into *NdeI*/*XhoI* digested pET-30b(+) vector. The following reaction conditions were applied for the PCR reaction containing 100 pg template DNA. Initial template DNA denaturation at 94°C for 5 min, 30 cycles at 94°C for 1 min, at 52°C for annealing, at 72°C for 1 min (synthesis), and at 72°C for 7 min of final extension.

Cloning and Expression of Bacteriophage Endolysin Genes

The PCR products were cloned into two different protein expression vectors using T4 DNA ligase. i) the endolysin gene fragment with a -T overhang at both ends was directly into pET SUMO expression vector (Invitrogen, USA) following manufacturer's recommandations. ii) the PCR product carrying the endolysin gene was first cloned into pJET 1.2 PCR cloning vector (Thermo Scientific, USA), and endolysin gene was removed from this vector with *NdeI*/*XhoI* double digest then cloned into pET-30b(+) expression vector (Thermo Scientific, USA), digested with the same enzymes. The recombinant plasmids carrying the endolysin genes were first transformed into *E. coli* One Shot MachlTM-T1R (Invitrogen, USA) competent cells. The recombinant plasmids were confirmed by DNA sequence analysis using a Genetic Analyzer 3130 (Applied Biosystems, USA). The recombinant plasmids were transferred into *E. coli* BL21 (DE3) competent cells (Invitrogen, USA) and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for expression at 20°C. Briefly, the transformed *E. coli* BL21 were grown in 5 mL of LB broth containing 50 µg/mL kanamycin at 250 rpm shaking overnight. The flowing morning 4 mL from overnight culture was seeded into 400 mL LB broth medium containing 50 µg/mL kanamycin, and induced with 1 mM IPTG when the culture OD_{600nm} reached 0.5-0.6.

Purification of Recombinant Endolysins

Purification processes of the endolysin protein were performed with the proBond Purification system kit (ThermoFisher, USA) in line with the manufacturer's recommendation. Briefly, cells were collected by centrifugation and were resuspended in 4-8 ml of lysis buffer (50 mM K_2HPO_4 pH 7.8, 400 mM NaCl, 100 mM KCl, 10 % glycerol, 0.5 % Triton X-100, 10 mM imidazole), followed by three cycles of freeze-thawing with alternating -20°C and 42°C. The cells were further disrupted by sonication for 10-sec sonication followed by 10-sec rest over 3 min (Vibra Cell™ Sonics, USA). The lysate was centrifuged at a maximum of 12,000 rpm for 10 min at 4°C to remove the cell debris. The supernatant was transferred to a sterile tube. The cell lysate was analyzed for the presence of expressed endolysins by SDS-PAGE analysis following the standard procedures. The endolysin-containing cell lysate was transferred to a purification column containing the nickel resin and further purified. Following the wash steps the endolysins were eluted with 2-8 mL elution buffer (50 mM Tris-HCl pH 8.00). The eluted endolysins were further concentrated using Pierce™ Protein Concentrators, 30K molecular weight cutoff (MWCO) (ThermoFisher, USA). The purity of the recombinant endolysin was checked by SDS-PAGE analysis, and the concentration of the endolysins was determined with the Qubit TM Protein Assay Kit (Invitrogen, USA) following the manufacturer's recommendations. The endolysins were sterilized using 0.22 µm pore-sized filters, and stored at -20°C until use.

Functional Analysis of Recombinant Endolysins

The lytic activities of the recombinant endolysins were tested by drop assay on the agar plate seeded with susceptible staphylococci. After 24 hours of incubation at 37°C, the formation of inhibition zones in the areas where the enzyme was dropped indicated the lytic activity. A turbidity reduction assay was performed in a 96-well microplate. Briefly, 200 µL of overnight cultures were transferred to 96-well microplate wells in triplicate. Then, the endolysin mix was added to the wells at various concentrations and incubated at 37°C for 60 minutes. After incubation, the bacterial concentration in the wells was determined by measuring the decrease in OD_{600nm}.

Anti-biofilm Activities of Endolysins

Crystal Violet Assay for Biofilm Quantification: Three test strains (*S. aureus*, *S. epidermidis*, and *S. haemolyticus*) strains were grown overnight in TSB medium containing 1% glucose (TSBG) and diluted 1/100, 200 µL was transferred to the wells of a 96-well microplate. The cell was allowed to form biofilm for 24-48 hours. Following the biofilm formation, the microwells were washed twice with PBS, followed by the addition of an equal amount of enzymes with varying concentrations, and incubated at 37 °C for 12 hours. The wells were washed twice with PBS and stained with 200 µL of 0.1% crystal violet for 5-10 min at room temperature. The wells were washed twice with distilled water, and the biofilm was dissolved in 200 µL of 95% ethanol and read in a microplate reader at OD_{595nm}. The amount of biofilm removed by endolysin was determined by comparison with the wells treated with PBS. Experiments were repeated at least three times.

Confocal Microscope Demonstration of Anti-biofilm Activities of Endolysins: Biofilms formed on an eight-chamber coverglass (Thermo Fisher Scientific, USA) were treated with endolysin for 12 hours. Then living and dead bacteria were stained with BacLight LIVE/DEAD (Invitrogen/Thermo Scientific, USA) according to the manufacturer's instructions and examined in three dimensions with Leica DMI8 Confocal Laser Scanning Microscopy with 63x objective (CLSM, Leica Microsystems, Germany). For imaging, the wavelength was set at 485/498 nm for SYTO 9 (fluorescent green) and at 535/617 nm for propidium iodide (fluorescent red) for the observation of live and dead bacteria, respectively.

RESULTS

PCR Screening of Phage Endolysin Genes in the S. aureus Genome

The chromosomal DNA from 173 *S. aureus* strains was included in the study as a template (Figure 1) for PCR amplification of endolysin genes of prophages, which are integrated into the chromosomes. The endolysin encoding gene about 1.5 kb in size, was amplified by PCR from the chromosome of six strains, TSA 2, TSA 4, TSA 5, TSA 6, TSA 22, and TSA 23 (Figure 2). The six endolysin genes, lys 2, lys 4, lys 5, lys 6, lys 22, and lys 23 were gel purified using Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit (Macherey-Nagel, Germany) and cloned into plasmid vectors.

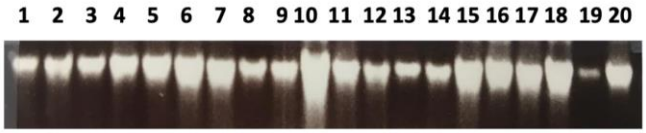

Figure 1. Agarose gel electrophoresis of chromosomal DNA from some *S. aureus* strains.

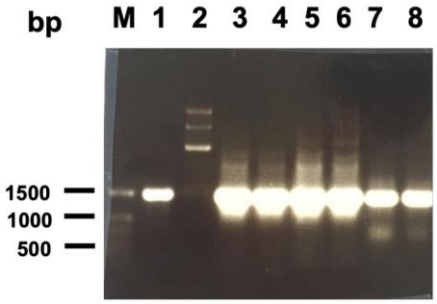

Figure 2. Amplification of phage endolysin genes from the chromosome of *S. aureus* strains by PCR. M; molecular weight standard, 1; pozitif control, 2; negative control, 3; lys 2, 4; lys 4, 5; lys 5, 6; lys 6, 7; lys 22, 8; lys 23.

Cloning of Phage Endolysin Genes

The gel prified PCR products were cloned into pET SUMO vector for expression without pre-cloning, and the pJET 1.2 PCR cloning vector before being cloned into the the pET-30b(+) vector for expression. Among six purified endolysin genes only endolysin gene 6 (lys 6) was successfully cloned into the pET SUMO vector for expression, and five endolysin genes (lys 2, lys 4, lys 5, lys 22, and lys 23) were cloned into pJET 1.2 vector (Figure 3), which were confirmed by restriction endonuclease digestion (Figure 4) before subcloned into pET-30b(+) expression vector.

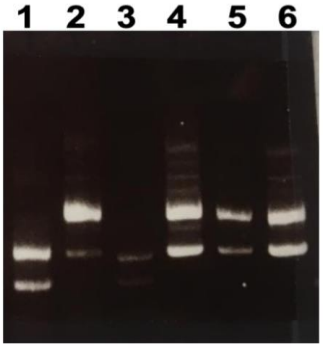

Figure 3. Undigested recombinant pJET 1.2 plasmids containing the endolysin genes. 1; control (pJET 1.2 vector without insert), 2; pJET 1.2_lys 2, 3; pJET 1.2_lys 4, 4; pJET 1.2_lys 5, 5; pJET 1.2_lys 22, 6; pJET 1.2_lys 23.

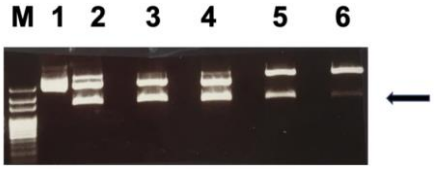

Figure 4. Confirmation the presence of endolysin genes cloned into pJET 1.2 vector by cleavage with *NdeI* and *XhoI* restriction enzymes. M; molecular weight standard, 1; pJET 1.2_lys 2 uncut, 2; pJET 1.2_lys 2 cut with *Nde*I and *Xho*I, 3; pJET 1.2_lys 4 cut with *Nde*I and *Xho*I, 4; pJET 1.2_lys 5 cut with *Nde*I and *Xho*I, 5; pJET 1.2_lys 22 cut with *Nde*I and *Xho*I, 6; pJET 1.2_lys 23 cut with *Nde*I and *Xho*I. The arrow indicates 1.5 kb endolysin gene.

The endolysin genes from pJET 1.2 vector were excised with NdeI and XhoI restriction enzymes and cloned into the pET-30b(+) vector, which was cut with the same enzymes. Except lys 22, lys 2, lys 4, lys 5, and lys 23 genes were successfully subcloned into pET-30b(+) plasmid vector, and confirmed by restriction endonuclease analysis and sequencing.

Recombinant Production of Endolysins

The expression vectors containing the endolysin genes were expressed in *E. coli* BL21(DE3) and obtained in soluble form from the cell lysates of 250-1000 mL cultures. The high enzyme yield with activity was obtained if the cells were induced at a cell density of 0.5-0.6 at OD_{600nm}, with 1 mM IPTG addition, and at 22-24°C incubation for 18-22 hours. The presence of dissolved endolysins was detected by SDS-PAGE analysis, and those with lytic activity from these crude lysates were purified by passing them through nickel columns (Figure 5).

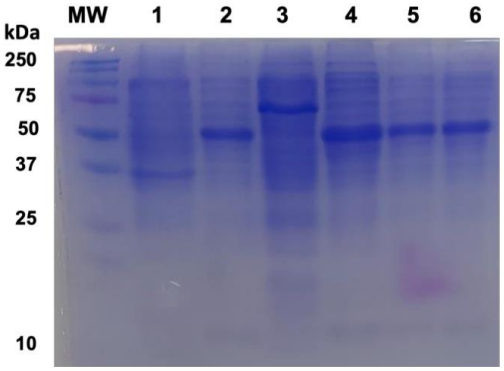

Figure 5. SDS-PAGE analysis of recombinant endolysins. MW; molecular weight standard, 1; pET SUMO_lys 6 uninduced culture lysate, 2; pET-30b(+)_lys 2, 3; pET SUMO_lys 6, 4; pET-30b(+)_lys 4, 5; pET-30b(+)_lys 5, 6; pET-30b(+)_lys 23.

Functional Analysis of Endolysins

Anti-staphylococcal activities of recombinant endolysins were tested against three different clinical *Staphylococcus* species (*S. aureus, S. epidermidis* and *S. haemolyticus*) in our collection using the agar-drop assay (Figure 6).

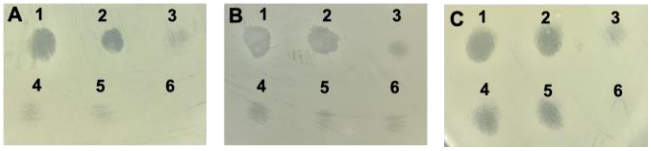

Figure 6. Demonstration of antibacterial activities of recombinant endolysins by agar-drop assay. A; *S. aureus* TRSA 2, B; *S. epidermidis* TRSE 6, C; *S. haemolyticus* TRSH 1, 1; Control (pET SUMO_lys10), 2; pET SUMO_lys 6, 3; pET-30b(+)_lys 2, 4; pET-30b(+)_lys 5, 9; pET-30b(+)_lys 23.

It was determined that both vectors (pET-30b(+) and pET SUMO) functionally produced recombinant phage endolysins in *E. coli*. However, these recombinantly produced endolysins were obtained relatively low concentrations ranging from 0.4 to 1 mg/L using shake flask cultures. Therefore, turbidity reduction, anti-biofilm, and biofilm reduction tests were carried out using a mixture five endolysins at 1-5 µg/mL after further concentration.

Turbidity Reduction

The turbidity reduction test was performed to demonstrate the antibacterial activity of endolysins (33). The reduction in bacterial cell concentration was measured at OD_{600nm} and plotted against time (Figure 7). The growth of three different species of staphylococci was inhibited approximately 50% indicated by the less cell density after 60 minutes compared to the controls.

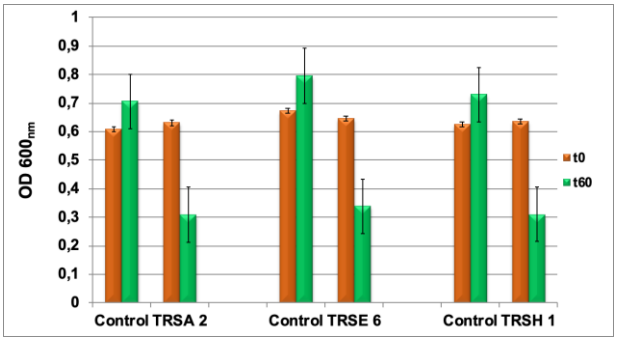

Figure 7. Demonstration of anti-staphylococcal activity of endolysins.

Anti-biofilm Activities of Endolysins

Crystal Violet Method: The *Staphyloccus* strains were grown in TSB medium (TSBG) containing 1% glucose overnight. The cultures were diluted 100 fold with TSBG medium and 200 µL was transferred to microplates with 96 wells, and a biofilm was formed for 24-48 hours as described before.[12] Following biofilm formation, the microwells were washed twice with PBS, followed by the addition of an equal amount of PBS containing varying concentrations of endolysin, and incubated at 37°C for 12 hours. Following the incubation, the wells were washed twice with PBS and stained with 200 µL of 0.1% crystal violet for 5-10 min at room temperature. The wells were washed twice with distilled water, biofilm was dissolved in 200 µL of 95% ethanol, and dissolved crystal violet was read in a microplate reader at OD_{595nm}. The amount of biofilm removed by endolysin was determined by comparison with wells treated with PBS. Experiments were repeated at least three times (Figure 8).

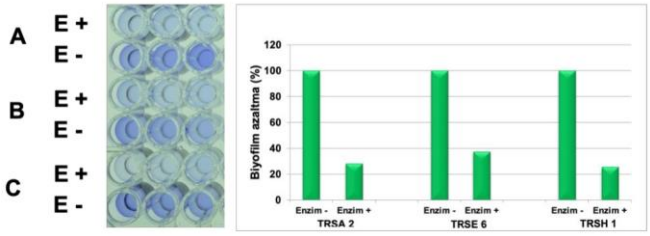

Figure 8. Removal of biofilms by endolysins. A; *S. aureus* TRSE 2, B; *S. epidermidis*, C; *S. haemolyticus*, E+; Endolysin added, E -; Endolysin not added.

Confocal Microscopy Method: Biofilms formed on the eight-well special glass coverslips were treated with endolysin for 12 hours. Then living and dead bacteria were stained with BacLight LIVE/DEAD and examined in three dimensions with Leica brand Confocal Laser Scanning Microscopy. Live bacteria were stained green with SYTO 9 dye and dead bacteria were stained red-orange with propidium iodide, and the biofilm removed by endolysin was evaluated qualitatively (Figure 9). The endolysin-treated biofilms from three different species of *staphylococci* were stained red indicating the death of the cells as compared to untreated biofilms. The rate of anti-biofilm activity was higher with the increase in endolysin concentration (Figure 9). Among all four strains, biofilms or biomass reduction of *S. aureus* TRSA 2, and *S. haemolyticus* TRSH 1 seemed to be higher by the lytic activity of the endolysins.

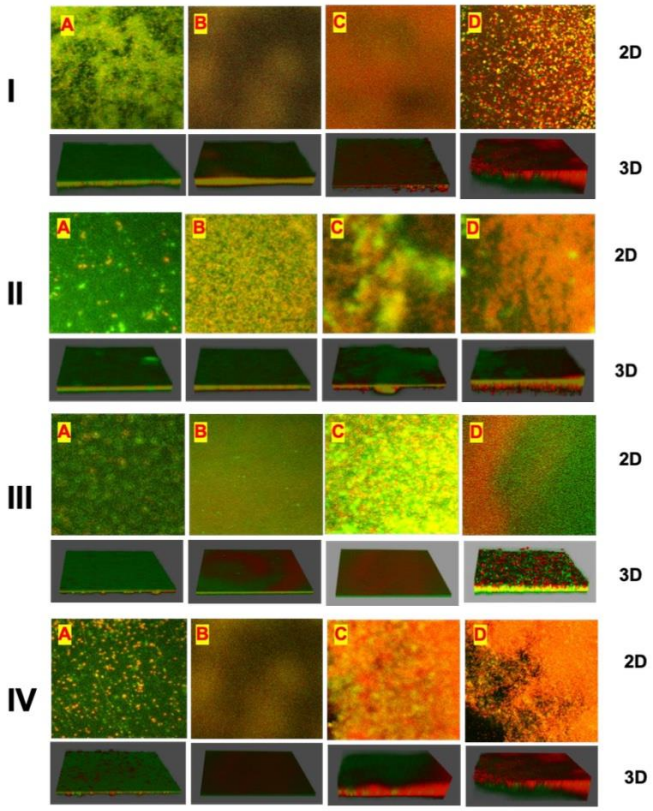

Figure 9. Demonstration of the anti-biofilm property of endolysin by confocal microscopy. Two (2D) and three-dimensional (3D) confocal microscopy (X630) of the concentration-dependent effects of recombinant endolysins on biofilms of *staphylococcal* strains. I; *S. aureus* TRSA 2, II; *S. aureus* TRSA 8, III; *S. epidermidis* TRSE 6, IV; *S. haemolyticus* TRSH 1, A; no endolysin, B; 1 µg/mL endolysin, C; 2.5 µg/mL endolysin, D; 5 µg/mL endolysin.

DISCUSSION

In this study, endolysin proteins of phages infecting *Staphylococcus aureus* strains isolated from various foods and personnel working with these foods were produced recombinantly in *E. coli*, and the potential anti-staphylococcal and anti-biofilm properties of these enzymes were observed in vitro. Here we show that *staphylococci* carry prophages widely and these prophages are suitable sources for endolysins and pET SUMO and pET-30b(+) vectors are suitable for cloning and recombinant production of endolysin genes. Although pET- series and several other vectors were used for the expression of phage endolysins, SUMO fusion was rarely used for this purpose. In this study, endolysin proteins of phages infecting *S. aureus* strains isolated from various foods and personnel working with these foods were produced recombinantly in *E. coli*, and the potential anti-staphylococcal and anti-biofilm properties of these enzymes were observed in vitro. Here we show that staphylococci carry prophages widely and these prophages are suitable sources for endolysins and pET SUMO and pET-30b(+) vectors are suitable for cloning and recombinant production of endolysin genes. A number of parameters have been used to increase the yield of recombinant endolysins. Neither the solubility tags nor co-expressed molecular chaperones were effective. Only significant yield improvement resulted from the induction temperature, and lysis buffer additives.[22] In this study, endolysins expressed in both pET SUMO and pET-30b(+) had indistinguishable SDS-PAGE band intensities and lytic activities, Figure 5 and Figure 6, respectively.

The growth of cultures in the presence of endolysin was found to be reduced by approximately 50% after 60 minutes compared to the control (Figure 7) as indicated by the turbidity reduction. Similarly, in the crystal violet method, biofilms formed with *S. aureus* TRSA 2, *S. epidermidis* TRSE 6, and *S. haemolyticus* TRSH 1 strains were reduced by at least 70% compared to the control biofilm without endolysins (Figure 8). The reducing activity of the endolysins, viable and dead cells was visible using confocal laser scanning microscopy, which is one of the valuable tools for biofilm studies.[23] The use of confocal microscopy not only provides the 3D structure of the biofilm but also provides valuable information about the penetration and action of the endolysin through the biofilm (Figure 9).

Although there are several limitations in this study, such as growing *E. coli* cultures in shake flasks in limited volumes instead of liters amount in more controlled fermentors, due to limited resources not being able to optimize the culture, induction, and cell lysing conditions, most of all not being able to obtain endolysins in high yield and purity.

The potential use of phage endolysins to prevent or remove both gram-positive and gram-negative bacterial biofilms has been reviewed in several recent studies. [24, 25, 26, 27, 28] Lysins act on the peptidoglycan layer of bacterial cell wall at the end of lytic cycles of the phages from within. Since the activity of lysin is compromized by the bacterial outer cell membrane of Gram-negative bacteria, membrane permeability to lysins are achieved by addition of membrane distabilyzing agents or fusion of lysin gene with a highly hydrophobic amino acid residue coding genes. In this study, lysin was expressed in its native form and was active only against members of staphylococci. There have been numerous recombinant phage lysins some of which been in clinical trials, for the treatment of bloodstream infections such as bacteremia and endocarditis. This is the first report on the phage endolysins from staphylococcal temperate phages from Türkiye.

In this work, endolysin encoded by a temperate bacteriophage in the chromosome of staphylococci showed hydrolytic activity against three species of staphylococci. A similar study was reported by phage DW2 from a bovine mastitis isolate of *S. aureus*.[29] These results indicate that functional endolysins could be cloned and recombinantly produced from both lytic and temparate phages, which increase the rate of finding ptentially broad host-range lysins.

It is noteworthy that endolysin genes originating from *S. aureus* strain and produced recombinantly in *E. coli* were able to lyse other species of staphylococci, most probably other relative gram-positive bacteria, which deserves further studies.

CONCLUSION

In this study, four phage endolysin genes belonging to the temperate phages integrated into the chromosomes of *S. aureus* strains, isolated from nose and hands, and food processing surfaces were amplified, cloned, and recombinantly expressed in *E. coli*. Their potential to be used as “enzybiotics” were demonstrated against different species of staphylococci by anti-bacterial, biofilm prevention, and biofilm removal experiments.

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Authorship contribution statement

Consept and desing: AOK.

Acquisition of data: SP, OBO, ID, UU.

Analysis and interpretation of data: AOK, SP and EK.

Drafting of the manuscript: AOK and SP.

Critical revision of the manuscript for important intellectual content: OBO and EK.

Statistical analysis: SP.

Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

Ethical approval

Ethics committee approval is not required.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Case Report

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Hemodialysis, Levocarnitine and Meropenem Application in the treatment of severe Valproic Acid-Induced Toxicity in the Intensive Care Unit

Yoğun Bakım Ünitesinde Valproik Asit Kaynaklı Ciddi Toksisite Tedavisinde Hemodiyaliz, Levokarnitin ve Meropenem Uygulaması

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ABSTRACT

Valproic acid (VPA) is currently a drug that can be used in the prophylaxis of epilepsy, bipolar disorder, schizoaffective disorder, schizophrenia and migraine. Valproic acid poisoning can result in central nervous system depression, shock, acute hyperammonemia, cerebral edema, coma and death. In this case, we wanted to present a patient who was treated with levocarnitine, hemodialysis and meropenem due to severe VPA toxicity. A 41-year-old male patient was firstly brought to the emergency room and then Intensive Care Unit with confusion. The patients was intubated because of his Glasgow Coma Score of 6 and respiratory failure. The patient had tachycardia, hypotension, and needed vasopressor medication. While liver function tests were found to be normal, hyperammonemia was thought to be drug-related. The patient's blood valproate level was 1055mg/L (therapeutic normal range: 50-100mg/L). The patient who developed aspiration pneumonia was given meropenem, which was found to be beneficial in valproic acid intoxication. Clinical manifestations of valproic acid poisoning are central nervous system depression, respiratory depression, hypotension, metabolic acidosis, bone marrow failure, brain edema, hypoglycemia, hypernatremia, and hyperammonemia. Treatment of acute valproic acid poisoning usually includes supportive care and fluid therapy, vasopressor support, electrolyte imbalance, and correction of acid-base disorders. Since the initial valproate level was 1055 uq/ml in our case, we applied to hemodialysis as the first treatment. We used levocarnitine especially because it can be useful in hyperammonemia. As a result, we observed that combined treatment of hemodialysis, levocarnitine and meropenem rapidly decreased the valproic acid level and resulted in a successful recovery.

Keywords: Valproic acid, Intoxication, Levocarnitine, Dialysis, Intensive Care.

ÖZET

Valproik asit (VPA) günümüzde epilepsi, bipolar bozukluk, şizoafektif bozukluk, şizofreni ve migren profilaksisinde kullanılabilen bir ilaçtır. Valproik asit zehirlenmesi merkezi sinir sistemi depresyonu, şok, akut hiperammonemi, beyin ödemi, koma ve ölümle sonuçlanabilir. Bu olguda şiddetli VPA toksisitesi nedeniyle levokarnitin, hemodiyaliz ve meropenem tedavisi uyguladığımız bir hastayı sunmak istedik. 41 yaşında erkek hasta önce acil servise sonrasında Yoğun Bakım Ünitesi'ne bilinç bulanıklığıyla getirildi. Glasgow Koma Skoru 6 ve solunumu yetersiz olduğu için entübe edildi. Hastanın taşikardisi, hipotansiyonu, vasopressor ilaç ihtiyacı vardı. Karaciğer fonksiyon testleri normal bulunurken, hiperammoneminin ilaca bağlı olduğu düşünüldü. Hastanın kan valproat düzeyi 1055mg/L (terapötik normal aralık: 50-100mg/L) bulundu. Aspirasyon Pnömonisi gelişen hastaya valproik asit intoksikasyonunda yararlı olduğu saptanan meropenem verildi. Valproik asit zehirlenmesinin klinik belirtileri, merkezi sinir sistemi depresyonu, solunum depresyonu, hipotansiyon, metabolik asidoz, kemik iliği yetmezliği, beyin ödemi, hipoglisemi, hipernatremi ve hiperamonyemidir. Akut valproik asit zehirlenmesinin tedavisi genellikle destekleyici tedavi ve sıvı tedavisi, vazopresör desteği, elektrolit dengesizliği ve asit-baz bozukluklarının düzeltilmesini içerir. Olgumuzda başlangıç valproat düzeyi 1055 uq/ml olduğu için ilk tedavi olarak hemodiyalize başvurduk. Levokarnitin özellikle hiperammonemide yararlı olabileceğinden kullandık. Sonuç olarak hemodiyaliz, levokarnitin ve meropenem kombine tedavisinin valproik asit düzeyini hızla düşürdüğü ve başarılı bir şekilde iyileşme sağladığını gözlemledik.

Anahtar Kelimeler: Valproik asit, İntoksikasyon, Levokarnitin, Diyaliz, Yoğun bakım.

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INTRODUCTION

Valproic acid (VPA) is a drug that can be used for prophylaxis of epilepsy, bipolar disorder, schizoaffective disorder, schizophrenia and migraine today.[1] Side effects related to VPA use are nausea, diarrhea, vomiting, pancreatitis, leukopenia, anemia, thrombocytopenia, hypofibrinogenemia, metabolic acidosis, hypernatremia, hypocalcemia, hypoglycemia, acute renal failure, hypothermia, hypotension and tachycardia.[2,3] Valproic acid intoxication may result in central nervous system depression, shock, acute hyperammonemia, brain edema, coma and death.[4] There is no specific antidote for valproate intoxication.[5] The treatment is supportive therapy. Traditional treatment methods have been defined as activated charcoal, levocarnitine and hemodialysis. Studies have shown that naloxone is useful in carbapenem antibiotics. However, none of them came to the fore as standard treatment.[6,7]

In this case report, we present a case in which we applied levocarnitine, hemodialysis and meropenem treatment due to severe VPA toxicity in the Intensive Care Unit.

CASE REPORT

A 41-year-old male patient with a diagnosis of schizophrenia and hypertension was brought to the emergency department and then in the Intensive Care Unit. When the patient admitted to the Emergency department, he was intubated because his Glasgow Coma Scale (GCS) was 6. There was no abnormality in the patient's vital signs, except for sinus tachycardia. Vasopressor support was provided to the patient who developed deep hypotension during the follow-up (norepinephrine 0.48 mcg/h). The patient's laboratory data are summarized in Table 1. He was found to be taking 50 g valproate. The patient's blood valproate level was found to be 1055 mg/L (therapeutic normal range: 50-100 mg/L). The change in the patient's valproate levels is shown in Figure 1. While liver function tests were normal, hyperammonemia was thought to be drug-related. No edema was detected in brain tomography. Activated charcoal treatment was not given to the patient whose medication time was uncertain. Due to hyperammonemia, 6 g bolus L-carnitine, was given as maintenance 9 g/day. Hemodialysis (HD) was taken 3 times until the valproate level fell below 100 mg/L. Every 6 hours, valproic acid, Artreial Blood Gases (AKG), Complete Blood Count (CBC), electrolyte, Liver Function Tests (LFT), ammonia, and ECG were monitored. The followed parameters of the patient are presented in Table 1. The patient who developed pneumonia due to aspiration was given meropenem, which was found to be beneficial in valproic acid intoxication. The patient's clinical improvement was extubated on the 5th day. The patient, whose general condition improved, was transferred to the psychiatry service.

Table 1. The followed parameters of the patient

Paramaters	**1 DAY**	**2 DAYS**	**3 DAYS**	**4 DAYS**	**5 DAYS**
Glucose (mg/dL)	129	119	162	128	261
Creatinine (mg/dL)	0.9	0.8	0.81	0.91	0.68
ALT (IU/L)	18	14	12	35	76
Sodium (mmol/L)	142	148	139	144	138
Potassium(mmol/L)	3.8	3.5	3.4	3.6	3.3
Amylase	171	155	65	49	75
pH	7.38	7.41	7.52	7.36	7.37
PCO_2 (mmHg)	36.6	41.1	32.5	36.4	32.7
PO_2 (mmHg)	103	159	164	147	93
O_2 support	mechanical ventilation	mechanical ventilation	mechanical ventilation	mechanical ventilation	extubated
Lactate(mmol/L)	14	17	12	7	8
HCO_3^- (mmol/L)	22.4	25.9	25.6	24.1	20.1
Leukocytes (10^3/µL)	6.25	7.8	11.4	10.23	6.96
Hemoglobin (g/dL)	15.6	13.1	12.5	11.3	10.8
Platelet(160x10^3uq/L)	160	153	79	64	108
Valproic Acid (mg/L)	1055	530	127	76	5
Ammonia	56	106	78	714	92
Need for vasopressors	+	-	-	-	-

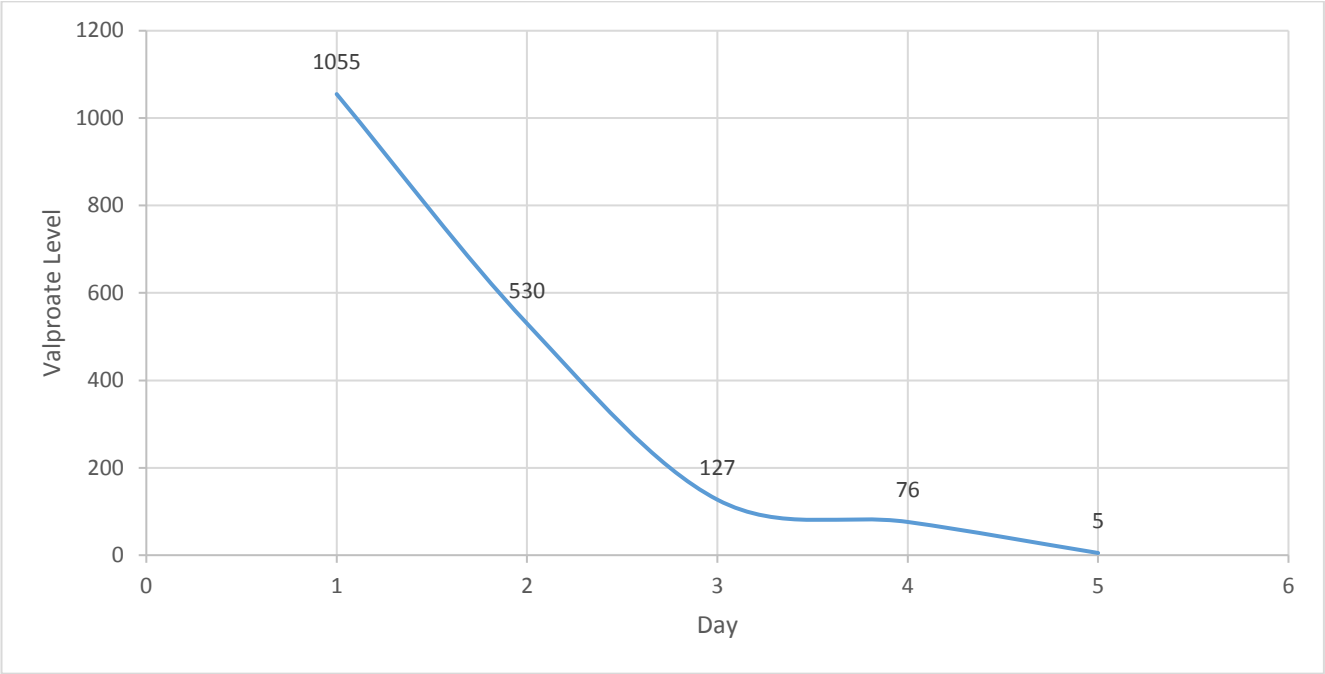

Figure 1. *The change in the patient's valproate levels.*+-

DISCUSSION

Valproic acid poisoning can manifest with various symptoms and is directly related to its plasma concentration. Serious poisonings usually occur above 850 uq/mL and can be life-threatening.[1] Clinical signs of valproic acid intoxication are central nervous system depression, respiratory depression, hypotension, metabolic acidosis, bone marrow failure, cerebral edema, hypoglycemia, hypernatremia, and hyperammonemia.[8] CNS depression usually plasma concentration 180 uq/mL It is observed when it exceeds. Thrombocytopenia is observed when the plasma concentration exceeds 450 uq/mL.[9] Our patient had central nervous system depression, respiratory depression, hypotension, thrombocytopenia, hypernatremia, and hyperammonemia. Activated charcoal treatment may be beneficial in early admissions. In our case, activated charcoal was not used because the drug intake time developed for 12 hours. Treatment of acute valproic acid intoxication usually includes supportive therapy and fluid therapy, vasopressor support, electrolyte imbalance, and correction of acid -base disorders. In addition, there may be a need for mechanical ventilation in consciousness disorders due to central nervous system depression or brain edema.[9] Levocarnitine may be especially useful in hyperammonemia. It is thought that the mechanism of action of levocarnitine may be effective in reducing the ammonia level and eliminating the coma picture. The recommended treatment dose of levocarnitine is 50-100 mg/kg/day.[10,11] Since our patient had hyperammonemia, recommended doses of levocarnitine were used.

Valproate level is above 850 uq/mL and severe neurological or cardiovascular symptoms are observeD.[12] Since the initial valproate level was 1055 uq/ml in our case, we applied to hemodialysis as the first treatment for the patient. After 3 sessions of hemodialysis, a significant decrease was found in serum valproate and ammonia levels.

Khobrani et al., were observed that the mental status of the patient improved despite the high ammonia level in which meropenem treatment was applied for aspiration pneumonia and valproate toxicity together with levocarnitine treatment.[13] Since our patient had aspiration pneumonia, meropenem antibiotherapy, which is known to be beneficial in valproate toxicity, was preferred as a treatment option. Figure 1 shows the decrease in valproic acid level after treatment.

CONCLUSION

As a result of the treatments, our patient was successfully extubated and discharged from the intensive care unit without any sequelae. As a result, it was observed that combined treatment of hemodialysis, levocarnitine and meropenem rapidly decreased the

valproic acid level and successfully treated the intoxication of it.

Authorship contribution statement

Consept and desing: CNDŞ, NY, HB, AE.

Acquisition of data: CNDŞ, NY, HB, AE.

Analysis and interpretation of data: CNDŞ, NY, HB, AE.

Drafting of the manuscript: AE.

Critical revision of the manuscript for important intellectual content: AE.

Supervision: AE.

Declaration of competing interest

None of the authors have potential conflicts of interest to be disclosed.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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İnternet Ortamında ve Sosyal Medyada Doğru ve Güvenilir Sağlık Bilgisi Edinebilme

Obtaining Accurate and Reliable Health Information on the Internet and Social Media

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ÖZET

Günümüzde internet, birçok alanda olduğu gibi sağlık alanında da bilgi edinebilmek için başvurulan ilk kaynak haline gelmiştir. Ancak internet ortamında herkes her istediğini yazıp yayımlayabilmekte, özellikle sosyal medyada çok kısıtlı bir kontrol dışında kişisel ve kurumsal yorumlar rahatlıkla yayılabilmektedir. Dolayısıyla internette, doğru bilgilerle birlikte genellikle yanlış veya doğrulanmamış aşırı bilgilerin hızlı bir şekilde yayılması durumu söz konusu olup bu durum "İnfodemi" olarak ifade edilmektedir. Böyle bir durum içerisinde, ulaşılan bilgilerin bilimsel ve çağdaş tıp uygulamaları açısından doğru olup olmadığının bilgiye ulaşan kişiler tarafından değerlendirilememesi önemli bir halk sağlığı sorunu olarak karşımıza çıkmaktadır. Yanlış bilgi kirliliği içinde karışıklığa düşmeden sağlık sorunlarına yönelik bilinçli kararlar alabilmek, bireylerin hem doğru ve güvenilir bilgiye erişebilme hem de yanlış, kusurlu veya yanıltmayı amaçlayan bilgiyi güvenilir olandan ayırt edebilme yeteneğine bağlıdır. Bu derlemede, infodemi, misenformasyon, dezenformasyon, malenformasyon ve bunların sağlık üzerine etkileri hakkında bilgi vermek; bu durumla mücadele kapsamında geliştirilen "İnfodemi yönetimi" ve "İnfodemiyoloji" kavramlarını açıklamak; bireylerin ve toplumun, hangi bilgiye güvenileceği ve neden güvenileceği hakkında bilgi sahibi olabilmelerinin, bilginin ve kaynakların güvenilirliği hakkında yargıda bulunabilmelerinin, kısaca bilgiyi değerlendirme yetkinliklerinin geliştirilmesi noktasında sağlık okuryazarlığının ve özellikle dijital sağlık okuryazarlığının önemini vurgulamak; internet ortamında ve sosyal medyada sağlık ile ilgili bilgilere ulaşmada dikkat edilmesi gereken noktalara değinmek amaçlanmıştır.

Anahtar Kelimeler: Dijital sağlık okuryazarlığı, İnfodemi, Sağlık bilgisi, Sağlık okuryazarlığı, Misenformasyon

ABSTRACT

The internet has become the first source used to obtain information in the field of health. However, on the internet, anyone can write and publish whatever they want, and personal and corporate comments can be easily spread, especially on social media, outside of very limited control. Therefore, there is a rapid spread of false or unconfirmed excessive information on the Internet, along with accurate information, and this is referred to as "Infodemic". In such a situation, it is an important public health problem that the people who reach the information cannot evaluate whether the information obtained is correct in terms of scientific and contemporary medical practices. Being able to make informed decisions about health problems without falling into confusion due to misinformation pollution depends on the ability of individuals to both access accurate and reliable information and distinguish false, defective or misleading information from reliable information. This review aims; to give information about infodemic, misinformation, disinformation, maleinformation and their effects on health; to explain the concepts of "Infodemic management" and "Infodemiology" developed within the scope of combating this situation; to emphasize the importance of health literacy and especially digital health literacy, for individuals and society to have information about what information to trust and why, to be able to make judgments about the reliability of information and resources, in short, to improve the competence of individuals and society in evaluating information; to mention the points that should be considered in accessing health-related information on the internet and social media.

Keywords: Dijital health literacy, Health information, Health literacy, Infodemic, Misinformation

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GİRİŞ

İnternet ortamında ve sosyal medyada sağlık bilgisi arama

Günümüzde internet, birçok alanda olduğu gibi sağlık alanında da hayal edilemeyecek düzeyde bir bilgi denizine, hiç olmadığı kadar kolay erişim sağlamaktadır. Bilgi toplumunda, kişilerin gerek kendi gerekse aile bireylerinin hastalıkları, hastalıkların risk faktörleri, hastalıklardan korunma yolları ve tedavi yöntemleri hakkında bilgi edinebilmek için internet ortamından ve sosyal medyadan yararlandıkları görülmektedir.[1,2] Bu kaynaklar aracılığıyla ulaşılan ve doğru kullanılan sağlık bilgileri; sağlığı etkileyen yaşam tarzı faktörleri, tarama ve erken tanı, hastalıklarla başa çıkabilme, semptomların yönetimi, aktif tıbbi karar vermeye dahil olma, farklı tedavi ve rehabilitasyon seçenekleri gibi konuları anlamada ve değerlendirmede oldukça önemlidir. Dolayısıyla doğru ve güvenilir bilgiye, doğru zamanda ve doğru formatta ulaşabilmek özellikle sağlığın söz konusu olduğu bu durumlarda yaşam kurtarıcı olabilmektedir.[3] Ancak ulaşılan bilgilerin bilimsel ve çağdaş tıp uygulamaları açısından doğru olup olmadığının bilgiye ulaşan kişiler tarafından değerlendirilememesi kaotik bir duruma neden olmakta ve önemli bir halk sağlığı sorunu olarak karşımıza çıkmaktadır. Bu derlemede, infodemi, yanlış bilgi kategorileri ve yanlış bilginin sağlık üzerine etkileri hakkında bilgi vermek; bu durumla mücadele kapsamında geliştirilen "İnfodemi yönetimi" ve "İnfodemiyoloji" kavramlarını açıklamak; bireylerin ve toplumun, hangi bilgiye güvenileceği ve neden güvenileceği hakkında bilgi sahibi olabilmelerinin, bilginin ve kaynakların güvenilirliği hakkında yargıda bulunabilmelerinin, kısaca bilgiyi değerlendirme yetkinliklerinin geliştirilmesi noktasında sağlık okuryazarlığının ve özellikle dijital sağlık okuryazarlığının önemini vurgulamak; internet ortamında ve sosyal medyada sağlık ile ilgili bilgilere ulaşmada dikkat edilmesi gereken noktalara değinmek amaçlanmıştır.

ANA METİN

İnfodemi ve yanlış bilgi kategorileri

Günümüzde internet ortamında herkes her istediğini yazıp yayımlayabilmekte, özellikle sosyal medyada çok kısıtlı bir kontrol dışında kişisel ve kurumsal yorumlar rahatlıkla yayılabilmektedir. Dolayısıyla internette, doğru bilgilerle birlikte genellikle yanlış veya doğrulanmamış aşırı bilgi yüklenmesi durumu söz konusudur. Literatürde doğru ya da yanlış bilginin hızlı ve geniş kapsamlı yayılmasını, yanlış veya kusurlu bilginin ise farklı boyutlarını ifade etmek üzere sunulan farklı tanım ve terimler bulunmaktadır.

"Enformasyon/bilgi" ile "epidemi/salgın" kelimelerinin bir araya gelmesiyle oluşturulan ve Dünya Sağlık Örgütü (DSÖ) genel direktörü Dr. Tedros'un, Şubat 2020'de yaptığı *"Biz sadece bir pandemiyle değil aynı zamanda bir infodemiyle de savaşıyoruz"* açıklaması ile küresel düzeyde gündeme gelmiş bir kavram olan infodemi, DSÖ tarafından *"bir hastalık salgını sırasında dijital ve fiziksel ortamlarda yanlış veya yanıltıcı bilgiler içeren çok fazla bilgi"* şeklinde tanımlanmaktadır.[4,5] Belirli bir konuda, büyük miktarda, güvenilir olmayan ve muhtemelen yanlış bilginin, son derece hızlı bir şekilde yayılması durumu söz konusudur. Bu durum kısaca "bilgi salgını" şeklinde de ifade edilmektedir. Genellikle insanlara yardım etmek amacıyla paylaşılan fakat bütünüyle doğruluk içermeyen ya da çeşitli finansal kazanımlar, siyasal çıkarlar ve statüler elde etme amacıyla korkulardan ve endişelerden beslenerek üretilen bilgiler, bu şekilde internet ortamında giderek yaygınlaşmaktadır.[6]

Yanlış bilgi, sağlıkla ilgili bir durum veya hastalığın, ortaya çıkışı, nedeni, tedavisi, yayılma mekanizması gibi pek çok konuda üretilip paylaşılabilmektedir. Bununla birlikte yanlış bilgi, iyi niyetle sunulan ancak yanlış veya kusurlu bilgi (misenformasyon) şeklinde olabileceği gibi kusurlu veya yanlış olduğu sunan kişi/kişiler tarafından bilinen, kasıtlı olarak yanıltma amaçlı yayılan bilgi (dezenformasyon) şeklinde de karşımıza çıkabilmektedir. Bunların yanı sıra tanımlanan bir diğer yanlış bilgi kategorisi ise "kötü bilgi/malenformasyon" olup bir kişiye, sosyal gruba, kuruluşa veya ülkeye zarar vermek için gerçeğe dayalı bilgilerin bozularak yayılması şeklinde tanımlanmaktadır. Böylece yanlış, eksik ve karmaşık bilginin farklı boyutlarını ifade etmek üzere farklı tanım ve terimler sunulmaktadır.[7,8] Her üçü de bireylerin yaşamlarının her yönünü etkileyebilen ve sağlıklarını tehdit edebilen boyuta ulaşabilmektedir.

Yanlış bilginin sağlık üzerine etkileri

İnternet ortamında ve sosyal medyada kimi doğru kimi doğrulanmamış kimi ise yanlış olan çok fazla bilgi mevcut olduğundan bu durum insanların ihtiyaç duyduklarında güvenilir kaynaklar bulmalarını ve karar verme süreçlerini zorlaştırabilmekte, hatta onları doğru karardan uzaklaştırabilmektedir. Normalde çok zor geliştirilen ya da değiştirilen davranış kalıpları ve

inanışlar, hızlı ve yoğun, yanlış ya da doğruluğu teyid edilmemiş bilgi akışı nedeniyle kolayca gelişebilmekte; özellikle sağlığın söz konusu olduğu stres altındaki dönemlerde davranışlar hızla değişebilmektedir. Dolayısıyla sağlık davranışları da dahil olmak üzere yaşamın her yönünü etkileyebilen yanlış bilgi, bireylerin doğru davranış ve tercihlerden uzaklaşıp risk altına girmelerine neden olabilmekte ve sağlıklarını olumsuz yönde etkileyebilmektedir.[9]

İnternet ortamından ve sosyal medyadan bilgi edinme yaklaşımı, faydalı olduğu iddia edilen ve bireyleri faydalı olduğu düşüncesine iten süreçlerin yaşandığı, ancak çoğu zaman olumlu etkileri yanında istenmeyen etkileri ortaya çıkabilen, konvansiyonel ilaçlarla farmakokinetik ve farmakodinamik düzeyde etkileşim nedeniyle çeşitli toksik etkilerin meydana geldiği ve hatta geri döndürülemeyen zararlara yol açabilen modern tıp dışı uygulamalara yönlendirmesiyle önemli sağlık sorunlarına yol açabilmektedir.[10-12]

Sağlık konusunda ilk bilgi kaynağı internet ortamı ve sosyal medya olan kişilerin, bu ortamlarda gördükleri veya okudukları hastalık tanılarını ve bu doğrultuda bedensel belirtilerini yanlış yorumlamaları, şiddetli sağlık kaygısı ya da diğer bir ifade ile "Hipokondriazis" olarak adlandırılan, ciddi bir hastalığa yakalanmış oldukları ya da yakalanacakları endişesi taşımalarına neden olmaktadır.[13,14] Bu kişiler, birinci, ikinci ve üçüncü basamak, özel ya da kamu farketmeksizin herhangi bir sağlık kuruluşuna başvurduklarında, *"Hocam, internette okuduğum bilgilerle ben şöyle bir hastalık olduğunu düşünüyorum…", "Hocam sosyal medyada gördüm. Şu ilaç çok iyi geliyormuş. Sen bana ondan yaz…*" şeklinde cümleler hekimler tarafından sıkça duyulmaktadır. Ancak doğrusu, hastalıkların hekim ile hasta arasında birlikte karar verilerek ve birlikte yönetilerek tedavi edilmesi olduğundan, bu düşünceler zaman zaman hekim-hasta iletişimini de olumsuz yönde etkilemektedir.

Sağlığın söz konusu olduğu bir durumla ilgili yanlış bilginin yayılması, etkili halk sağlığı müdahalelerini engelleyebilmekte, insanlar arasında kafa karışıklığı ve güvensizlik yaratabilmektedir. Bu süreç COVID-19 pandemi sürecinde derin bir şekilde yaşanmıştır.[15] COVID-19 pandemisi gibi aniden ortaya çıkan ve ortaya çıktığı andan itibaren gündemde yer alan bir sağlık olayı karşısında, insanlar bu yeni hastalık ve virüs hakkında bilgi edinmek, uygulama ve davranışlarını hızlı bir şekilde değiştirmek zorunda kalmışlardır. Ancak hızlı ilerleyen bu süreçte, alanında uzman olup olmadığı belli olmayan ya da salgın konusu ile birebir örtüşmeyen alanlarda uzman olan insanlarla, bilimselliği soru işareti taşıyan yaklaşımlar sergilendiği görülmüştür. Ortaya atılan komplo teorileri ve yapılan yanlış açıklamalar sonucunda bilginin doğruluğu, yanlışlığı, gerçekliği, bilimselliği ve uygulanabilirliği noktasında bilgi kirliliği meydana gelmiştir. Meydana gelen bu bilgi kirliliği, bireylerin, maske kullanımı, fiziksel mesafe ve aşı gibi sağlığı korumada en etkili olduğu kanıtlanan korunma yollarından uzaklaşmalarına ve salgından negatif yönde etkilenmelerine neden olmuştur.[9]

İnfodemi yönetimi ve infodemiyoloji

İnfodemi mücadelesi oldukça zor ve kapsamlı bir çaba gerektirmektedir. Ortaklıklar, paydaşlar ve iş birliği olmadan müdahale edilmesi ve sonuç alınabilmesi mümkün değildir. İnfodemi alanı, kişisel hak ve özgürlüklerin toplumsal çıkarları tehdit ettiği bir hal alabilmekte ve bu alan hukuk, ahlak, etik ve bilimin ortak zemini haline gelmektedir. Ayrıca infodeminin ulusal boyutu olmakla birlikte uluslararası ve hatta küresel boyutu daha da önem kazanmaktadır.[9] Bu bağlamda DSÖ tarafından "İnfodemi yönetimi" kavramı ortaya konmuştur. İnfodemi yönetimi, *"sağlıkla ilgili acil durumlar sırasında infodemiyi yönetmek ve sağlık davranışları üzerindeki etkisini azaltmak için risk ve kanıta dayalı analiz ve yaklaşımların sistematik kullanımı"* şeklinde tanımlanmaktadır. DSÖ'ye göre infodemi yönetimi ile; küresel düzeyde bilgi kontrolü, dezenformasyon ve misenformasyon izlemi ve yönetimi, infodemi ölçüm ve yönetimi, kanıt sentezi, bilginin anlaşılır hale getirilmesi, risk iletişimi, toplum katılımı ve mesajların güçlendirilmesi gibi faaliyetler aracılığıyla iyi sağlık uygulamalarına olanak sağlanması amaçlanmaktadır.[4] Ancak bu şekilde yanlış bilgi ve mesajların neler olduğu ve nasıl yayıldığı konusunda bilgi sahibi olunabileceği, doğru ve güvenilir bilgi oluşturulabileceği ve kişilere ulaştırılabileceği düşünülmektedir.

Bireylerin ve toplumun bilgi gereksinimlerini önceden belirlemek ve yanlış bilgiler yayılmadan doğru bilgileri ulaşılabilir hale getirmek oldukça önemlidir. Bu bağlamda yeni bir araştırma disiplini ve metodolojisi ortaya çıkmıştır. *"İnternetteki bilginin ve belirleyicilerinin dağılımı bilimi"* olarak tanımlanan "İnfodemiyoloji", bireyleri kaliteli sağlık bilgilerine yönlendirmede yararlı olabilecek sağlık bilgilerininin ve yanlış bilgilerin belirleyicilerini ve dağılımını incelemektedir. Ortaya çıktığı ilk zamanlarda yanlış, hatalı, zararlı enformasyonun analizi ve yönetimi

amacıyla geliştirilen infodemiyolojik yöntemler daha sonra internette dolaşan sağlıkla ilgili her türlü dijital veri ve enformasyonun izlenerek analizi yoluyla önemli halk sağlığı sorunları konusunda öngörüde bulunma amaçlı yöntemler olarak yaygınlaşmaya başlamıştır. Sonuç olarak nihai amacı halk sağlığı ve kamu politikasını bilgilendirmektir.[16-18]

Bilgiyi değerlendirme yetkinliği ve sağlık okuryazarlığı

Yanlış bilgi kirliliği içinde karışıklığa düşmeden sağlık sorunlarına yönelik bilinçli kararlar alabilmek, bireylerin hem doğru ve güvenilir bilgiye erişebilme hem de yanlış, kusurlu veya yanıltmayı amaçlayan bilgiyi güvenilir olandan ayırt edebilme yeteneğine bağlıdır. Ancak konuya nasıl yaklaşılacağı noktasında ciddi eğitim eksikliği mevcut olup bu eksiklik, özellikle gençlerin ve genç yetişkinlerin internet ortamında giderek daha fazla zaman harcadığı günümüzde toplum için büyüyen bir tehdit oluşturmaktadır. Dolayısıyla, bilginin ve kaynakların güvenilirliği hakkında yargıda bulunmak, hangi bilgiye güvenileceğini, neden güvenileceğini ve ne kadar güvenilebileceğini bilmek, kısaca bireylerin ve toplumun bilgiyi değerlendirme yetkinliğini geliştirmek temel bir gereksinim haline gelmektedir.[19-22] Bu bağlamda, son zamanlarda en çok tartışılan konulardan biri sağlık okuryazarlığı ve özellikle dijital sağlık okuryazarlığıdır.

"İnsanların sağlık, hastalığı önleme, sağlığın teşviki ve geliştirilmesi ile ilgili günlük yaşamda karar vermek ve kararlar almak için sağlık bilgilerine erişme, anlama, değerlendirme ve uygulama konusundaki bilgi, motivasyon ve yeterlilikleri" olarak tanımlanan sağlık okuryazarlığı, bireylerin sağlık bilgilerini anlamalarına ve bilinçli olarak sağlık kararları almalarına olanak sağlamaktadır.[23,24] Günümüzde sağlık sistemlerinin, sunulan hizmetlerden yararlanan bireylerden beklentisi, sağlık bakımındaki öz sorumluluklarının artırılması yönündedir. Dolayısıyla bireylerden kendi sağlık sorumluluklarını almaları, bilgiyi anlamaları, kendileri ve başkaları için doğru sağlık kararları vermeleri yönünde yeni roller üstlenmeleri istenmektedir. Tüm bu beklenti ve isteklerin temelinde ise kişilerin sağlık okuryazarlığı becerileri yer almaktadır. Dijital sağlık okuryazarlığı ise *"elektronik kaynaklardan sağlık bilgilerini arama, bulma, anlama, değerlendirme ve elde edilen bilgileri bir sağlık problemini ele almak veya çözmek için uygulama yeteneği"* şeklinde tanımlanmaktadır. Dijital sağlık okuryazarı olan bireyler, bir sağlık problemini çözmek için elektronik kaynaklardan bilgi alıp kullanabilen, bu bilgiler doğrultusunda kişisel sağlık ve bakım sorunlarını daha iyi yönetebilen kişilerdir. Dolayısıyla internet ve sosyal medya üzerinden kolaylıkla ulaşılabilecek sağlık bilgilerinin anlaşılması, yorumlanması, değerlendirilmesi ve yararlanılması gibi çözümler ile bireylerin dijital sağlık okuryazarlık seviyelerinin yükseltilmesi gerekmektedir. Yüksek dijital sağlık okuryazarlığına sahip bir bireyin, internet ortamında ve sosyal medyada herhangi bir bilgi ile karşılaşması durumunda, başlangıçtaki odağı; bilginin kaynağı, nasıl üretildiği ve kurumsal niteliği olmalıdır.[23,24]

Sağlık ile ilgili bilgilere ulaşmada dikkat edilmesi gereken noktalar

Bilginin kaynağını doğru seçmek ve bunun ötesinde o kaynağın saygınlığı ile güvenilirliğini değerlendirebilmek doğru ve güvenilir bilgiye ulaşmada oldukça önemlidir. Bu bağlamda dikkat edilmesi gereken önemli nokta bilgiyi sunan kişi/kişilerin alan uzmanlığı ve gösterilen kanıtların değeridir. Bilimin dilini veya diğer kültürel sembollerini kullanmak, bir insana uzmanlık atfedilmesi için yeterli değildir. Ayrıca bir alanda Nobel Ödülü sahibi olsa bile, bu o bilim insanının diğer alanlarda da uzman olduğu anlamına gelmemektedir. Ancak gerek internette gerekse sosyal medyada tüm bilim insanlarına "farklılaşmamış otoriteler" gözüyle bakılmaktadır. Dolayısıyla bireylerin, bilimde uzmanlığın belirteçlerini ve tüm bilim insanlarının aynı olmadığının önemini anlayabilmeleri gerekmektedir. Bununla birlikte alanında uzman kişi, bilimsel kanıtlara dayalı olarak görüşlerini ifade edebiliyorsa ve onlara uygun bir şekilde bir tanım yapabiliyorsa, ancak o zaman sunulan bilginin doğruluğu kabul edilmelidir.[22,25]

Uluslararası veya ulusal kongrelerdeki panellerde yapılan tartışmalar sonucu üretilen bilgiler; kongrelerdeki bildirilerde sunulan bilgiler; tıbbi çalışmalar neticesinde üretilen, yüksek kaliteli ve saygın bilimsel dergilerdeki makalelerde tanımlanan ve sunulan bilgiler, her ne kadar ortaya konma süreci uzun olsa da doğru ve güvenilir bilgiler olarak karşımıza çıkmaktadır. Dünya Sağlık Örgütü ve Birleşmiş Milletler Çocuklara Yardım Fonu (United Nations International Childrens Emergency Fund/UNICEF) gibi kuruluşlar başta olmak üzere, T.C. Sağlık Bakanlığı, devlet kurumları, uzmanlık kurumları, tıpta uzmanlık dernekleri, kanunla kurulmuş meslek örgütleri (Türk Tabipler Birliği/TTB, Türk Dişhekimleri Birliği/TDB, Türk Eczacılar Birliği/TEB vb.) tarafından sunulan bilgiler ise akut yani kısa süre içinde karşımıza çıkabilecek doğru ve güvenilir bilgi

kaynaklarıdır. Bu kurumsal bilgiler teyid edilme şansı ve olasılığı yüksek olan bilgilerdir. Dolayısıyla bireyler bu kaynaklardaki bilgilere daha fazla güvenebilirler.

SONUÇ

Günümüzde internet, faydalarının yanı sıra büyük bir yanlış bilgi salgınına da neden olmaktadır. Bu yanlış bilgi salgını, özellikle gençlerin ve genç yetişkinlerin internet ortamında giderek daha fazla zaman harcadığı günümüzde, bireyler ve toplum için büyüyen bir tehdit oluşturmaktadır. Bu tehdit karşısında öncelikle, internet ortamında ve sosyal medyada yazan her bilginin doğru kabul edilmemesi gerekmektedir. Bilginin kaynağı, nasıl üretildiği ve kurumsal niteliğinin olup olmadığı bireylerin öncelikli odak noktası olmalıdır. Kaynağı doğru seçmek ve bunun ötesinde o kaynağın saygınlığı ile güvenilirliğini değerlendirebilmek hiç olmadığı derecede önem arz etmektedir. Çünkü edinilen sağlık bilgisi kişilerin sağlıkla ilgili aldıkları kararları ve davranışlarını doğrudan etkileyebilmektedir.

Bireylerin ve toplumun kanıtları yorumlama yeteneği bilgiye bağlıdır ancak toplum bunun için gerekli bilgiden yoksundur. Dolayısıyla toplumun, doğru ve güvenilir sağlık bilgisinin nasıl elde edileceğine ve değerlendirebileceğine yönelik bilgilendirilmesi, bilgiyi değerlendirme kapasitesinin geliştirilmesi gerekmektedir. Kısacası bu noktada, sağlık okuryazarlığı ve dijital sağlık okuryazarlığı için gerekli yetkinlik oluşturulmalıdır. Çünkü bilginin çok hızlı ortaya çıktığı ve değiştiği bu çağda ancak bu yetkinlik kalıcı değere sahip olacaktır. Bu bağlamda ortaöğretim döneminden başlanarak, müfredatda yer alan "Sağlık Eğitimi" dersi kapsamında, sağlık bilgisi arama davranışı, doğru ve güvenilir sağlık bilgisine erişebilme, bilgiyi anlama ve değerlendirebilme ile ilgili içeriklere ağırlık verilmelidir. Hatta ilköğretim döneminde de ders veya seminerler kapsamında bir modül oluşturularak bu konulara yönelik eğitimler verilmelidir. Erişkin bireylerin ise yine çeşitli resmi ve özel eğitimler aracılığıyla sağlık okuryazarlığı ve dijital sağlık okuryazarlığı seviyeleri arttırılmalı, toplumda genel olarak dijital ortamda yayılan yanlış sağlık bilgileri konusunda farkındalık yaratılmalıdır.

Bireylerin dijital ortamda sağlık bilgisi edinme biçimlerini anlamak ve elde ettikleri verileri teyit ettikleri ya da doğruladıkları kanalları tespit etmek, sağlık okuryazarlığı çalışmaları kapsamında büyük önem arz etmektedir. Dolayısıyla bireylerin, sağlık bilgisine ulaşmak için hangi kanalları kullandıkları ve elde ettikleri bilgilerin doğruluğu ile ilgili ne ölçüde ve hangi biçimlerde araştırma yaptıkları konularında daha detaylı bilgi elde etmek için; yaş, gelir, bölge ve eğitim düzeyine göre geniş temsiliyet sağlayabilecek örneklemlerle çalışmalar yapılmalıdır. Hatta bu konuda derinlemesine görüşmeler ve odak grup görüşmeleri gibi nitel yöntemler kullanılarak da çalışmalar yapmak mümkündür. Ayrıca, infodemi ve sağlıkla ilgili yanlış bilgi sorunu tüm dünyayı ilgilendirdiğinden ve olumsuz etkilediğinden, uluslararası projeler yoluyla farklı ülkelerden veri toplayıp karşılaştırmalı çalışmalar yapmak literatüre önemli katkı sağlayacaktır. Bu çalışmalar sonucunda kullanıcıların dijital sağlık bilgisini arama ve onu teyit etme eğilimleri ölçümlendiğinde, doğru ve güvenilir sağlık bilgilerinin hangi kanallar vasıtasıyla dağıtılmasının toplumda karşılık bulacağı ve böylece sağlık okuryazarlığının geliştirilebileceği hakkında fikir yürütmek mümkün olabilecektir. Bu bağlamda, kamu sağlığı çerçevesinde kritik pozisyonda olan sağlık iletişimi için hangi mecraların/kişilerin daha "güvenilir ve saygın" olarak addedildiği tespit edilmeli ve sağlık mesajlarının iletiminde öncelikler belirlenmelidir. Sağlıkla ilgili kuruluşların resmi paylaşımlarını dijital kanallarda ön plana çıkartmak, dijital okuryazarlık programlarına ve haber doğrulama platformlarına kaynak sağlamak ve sosyal medya platformlarının dezenformasyona karşı önlemler almasını sağlamak atılabilecek önemli adımlardandır.

Bir diğer noktada ise hangi bilginin doğru hangi bilginin yanlış olduğunu değerlendirmesi gereken kişi her zaman okuyucu değildir. Önemli olan bireylerin ve toplumun bilgi gereksinimlerini önceden belirlemek, yanlış bilgiler yayılmadan doğru bilgileri ulaşılabilir hale getirmektir. Bu bağlamda bilim insanlarının sosyal medya başta olmak üzere çeşitli dijital platformlarda varlık göstermeleri, güncel araştırma sonuçlarını ve doğru olan bilimsel bilgiyi dolaşıma sokmaları gerekmektedir. Bu noktada Sağlık Bakanlığı tarafından, sağlık bilgisi edinmede en çok kullanılan kaynaklar olan internet ve sosyal medyada yer alan bilgilerin denetimi yapılmalıdır. Bakanlık tarafından, sağlık bilgisi veren sitelerin yeterliliğinin denetlenmesi, bir sertifikasyon sistemi kurulması sağlık konusundaki yoğun bilgi kirliliğinin önüne geçmede etkili olabilir. Bununla birlikte, yeterliliği kanıtlanmamış sitelerin kullanılmaması yönünde toplumu bilinçlendirici çalışmaların yapılması, geliştirilecek denetim sisteminin etkinliğini güçlendirebilir. Böylece bu ortamlarda var olan bilgilerin güvenilirliği, doğruluğu ve bilimselliği kontrol edilerek halka ulaştırılmış olacaktır. Ayrıca sosyal medya

kullanıcılarının doğru ve güvenilir sağlık bilgisi sunmaları için de yine Sağlık Bakanlığı tarafından "Sağlıkta Sosyal Medya Kullanım Kılavuzu" hazırlanmalıdır.

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Danışman: MT

Yazar çıkar çatışması

Yazarların arasında potansiyel çıkar çatışması yoktur.

Destek

Bu araştırma için maddi destek alınmamıştır.

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Medical Nutrition Therapy Approaches in Children with Autism Spectrum Disorder

Otizm Spektrum Bozukluğu Olan Çocuklarda Tıbbi Beslenme Tedavisi Yaklaşımları

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ABSTRACT

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that occurs in the early stages of development, characterized by limitations in social communication and interaction behaviors, repetitive limiting behaviors, and limited interests. Nutritional problems and gastrointestinal complaints seen in ASD have been known since the first diagnosis of the disease and are considered a feature of the disease. It is known that children with ASD have feeding problems five times more often than children with normal development. The most common nutritional problems seen in children with ASD are food selectivity, difficulty in eating skills, selective, strange, and unusual eating behavior, sensitivity to a particular presentation of food, avoidance of new foods, excessive and persistent intake of one type of food and meal time behavioral problems. Dietary approaches such as gluten-free, casein-free, ketogenic, special carbohydrate, Feingold, Candida body ecology, and eliminated allergy diets are applied. However, the evidence for these practices is limited. Therefore, it is recommended to monitor children with ASD in terms of inadequate and unbalanced nutrition and to consider feeding problems and malnutrition in applying restrictive and special diets.

Keywords: Autism, nutrition, diet

ÖZET

Otizm spektrum bozukluğu (OSB), gelişimin erken evrelerinde ortaya çıkan, sosyal iletişim ve etkileşim davranışlarındaki kısıtlamalar, tekrarlayıcı sınırlayıcı davranışlar ve sınırlı ilgilerle karakterize edilen nörogelişimsel bir bozukluktur. OSB'de görülen beslenme sorunları ve gastrointestinal şikayetler, hastalığın ilk tanısından itibaren bilinmekte ve hastalığın bir özelliği olarak kabul edilmektedir. OSB'li çocukların normal gelişim gösteren çocuklara göre beş kat daha fazla beslenme sorunu yaşadıkları bilinmektedir. OSB'li çocuklarda en sık görülen beslenme sorunları, besin seçiciliği, yeme becerisinde güçlük, seçici, garip ve sıra dışı yeme davranışı, belirli bir besin sunumuna karşı duyarlılık, yeni besinlerden kaçınma, bir tür besinin aşırı ve ısrarlı alımı ve yemek zamanı davranış sorunlarıdır. Glutensiz kazeinsiz diyet, ketojenik diyet, özel karbonhidrat diyeti, feingold diyeti, Candida vücut ekolojisi diyeti, elimine alerji diyetleri gibi beslenme yaklaşımları uygulanmaktadır. Ancak, bu uygulamalar için kanıt sınırlıdır. OSB'li çocukların yetersiz ve dengesiz beslenme açısından izlenmesi, kısıtlayıcı ve özel diyetlerin uygulanmasında beslenme sorunları ve yetersiz beslenmenin göz önünde bulundurulması önerilir.

Anahtar Kelimeler: Otizm, beslenme, diyet

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INTRODUCTION

Autism Spectrum Disorder (ASD) is characterized by significant impairment in social interaction and communication with restrictive and repetitive behaviors, interests, or activities.[1] ASD is a heterogeneous group of diseases that are difficult to diagnose and treat due to the lack of precise and clear information about the cause of the neurodevelopmental disorder.[2] Diagnostic criteria include three primary headings apparent inadequacy in social interaction, qualitative inadequacy in communication, and excessive struggle with repetitive interest.[3] ASD is a common neurodevelopmental disease with a rate of approximately 1.7%(1/59) in the United States.[4] The etiology of autism remains unclear. There are opinions that genetic and environmental factors are associated with autism symptoms.[5] Genetically based causes such as chromosomal abnormalities and gene defects constitute 10-20% of individuals with autism.[6] Environmental factors such as radiation, viruses, drug use, tobacco, alcohol, pesticides, endocrine disruptors, heavy metals, air pollution, and micronutrient deficiencies are also thought to play a role in ASD.[7,8] DSM-5 considers the clinical features of ASD in two dimensions as inadequacy social communication and repetitive ritual behaviors by bringing a dimensional approach. These clinical features begin in early childhood and adversely affect the activities of daily living. Recognizing these clinical features of ASD varies according to the severity of its symptoms, the child's developmental level, and age.[9] Deficiencies social communication include problems in social and emotional interaction, mutual dialogue, non-verbal communication, and establishing and conducting human relationships.[10] Restricted and repetitive interests, behaviors, and behavior patterns change with age and take on a different forms. Children with ASD tend to play repetitive games with non-functional objects, have repetitive conversations, show no interest in the typical toy and play, and examine or line them up rather than play with toys.[11]

Medical treatment of ASD

There is no pharmacological method that affects the basic symptoms of ASD. There are intervention methods applied to children with ASD in different approaches. These; evidence-based practices, comprehensive treatment models, alternative and complementary medicine (special nutrition interventions, vitamin-mineral supplements, etc.), and psychotherapeutic drugs.[12] Drug therapy is frequently used for comorbid psychiatric diagnoses, and behavioral problems accompanying ASD.[13] Treatment approaches are grouped as pharmacological and educational. The basic approach to alleviate disease-specific symptoms and increase functionality is educational therapies. Educational therapies aim to improve the field of social communication, reduce unwanted behaviors and gain new skills.[14]

Medical nutrition therapy of ASD

It has been observed that children with ASD are more exposed to parent-induced dietary restrictions, so parents of these children are more aware of the foods their children consume or should consume. It has also been observed that they make more effort to ensure that their children receive the recommended amount of nutrients.[15] In a cross-sectional study of children with autism, dietary intake of vitamins A, B_1, B_{12}, D, and folate, calcium, magnesium, potassium, iodine, omega-3, omega-6, linoleic acid, and α-linolenic acid was found insufficient.[16] In a study conducted on children with ASD, it was seen that there was suboptimal nutrient intake. Vitamin D, calcium, and bone health should be taken care of.[17] Vitamin B_{12} and vitamin D deficiency were significantly detected in children with ASD, and no significant difference in folate levels was found in healthy children.[18]

Energy

The metabolic and nutritional status of children with autism showed many differences when compared to healthy children. Children with autism have increased biomarkers indicative of oxidative stress and vitamin deficiency; energy transport, suffusion, and detoxification capacity are decreasing. Some of the biomarkers have been associated with autism severity.[19] In a study conducted on children with ASD, it has been observed that children with ASD between the ages of 2-5 are more prone to being overweight and obese, while children between the ages of 5-11 are more prone to being thin. In addition, it was found that children consumed a similar amount of nutrients and those with autism 4-8 years old children received less energy, zinc, and vitamins A and C.[20] No change in energy requirement has been reported in OSB.[21]

Carbohydrate

According to studies examining the intake of macronutrients in children with ASD, it has been shown that there is no significant difference compared to healthy children or that macronutrient intake is higher in

children with autism.[20-23] Although adequate intake of macronutrients versus insufficient intake of micronutrients is noteworthy, there is inconsistency in the results.[24] In some studies, it is reported that children with ASD have insufficient energy, carbohydrate, protein, and fat intake and that they take many vitamins and minerals inadequately due to their intestinal permeability and selective nutritional behaviors.[25-27]

Protein

5-20% of the daily energy intake for three years of age and 10-30% for four or six years of age should be provided from proteins. There needs to be more information about how much of the proteins that children receive should be of vegetable origin and how much of it should be of animal origin.[28] It has been shown that the protein intake levels of children with ASD are similar to those of typically developing children.[29]

The use of camel milk is seen to eliminate or reduce the behavioral problems seen in ASD. Camel milk, which does not contain beta-casein and beta-lactoglobulin, which can lead to cow's milk allergy, is a good source of protein. Camel milk, which is close to breast milk and rich in micronutrients such as calcium, phosphorus, niacin, and iron and strengthens natural immunity, has been thought to be used in the treatment of ASD.[30, 31] In a double-blind, randomized controlled trial, children aged 2-12 years were given camel milk for two weeks. Compared with the placebo group, significant improvements were observed in the Childhood Autism Rating Scale (CARS), Social Responsiveness Scale (SRS), and Autism Treatment Evaluation Checklist (ATEC) scores in children who received camel milk.[32]

Fat

Since omega-3 fatty acid deficiency is seen in children with ASD, it is thought that this deficiency may cause some symptoms caused by ASD and it is estimated that these symptoms will improve with omega-3 fatty acid supplementation as a supplement.[7] As a result of the examination of 143 articles written on this subject, it was concluded that there is not enough scientific evidence on the effectiveness and safety of the use of omega-3 fatty acid supplements for ASD.[33] In a study, 30 healthy children with ASD and 30 healthy children aged 3-11 years were examined and it was found that PUFA (Linoleic acid, linolenic acid, arachidonic acid, docosahexaenoic acid) levels were significantly lower in children with ASD. In the same study, children with ASD were given two capsules of omega 3 and omega 6 fatty acids twice a day for 3 months. As a result, improvements in PUFA levels and behavioral problems due to supplement use were observed in 20 children with ASD.[34] In another study, 12 individuals with ASD aged 18-40 years were given 0.93 g eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), 5 mg vitamin E fish oil capsules twice daily for 6 weeks. When the behavior of individuals is observed, it is concluded that behavior problems and the incidence of these problems are significantly reduced.[35] Fatty acid supplementation is thought to be associated with ASD and other neurological diseases. However, the number of randomized studies on this subject needs to be increased. Since the number of participants and the duration of the experiment vary in the studies conducted, it becomes difficult to interpret the results systematically.[36]

Vitamins

Levels of vitamins and minerals have been shown in many studies to be low in children with ASD.[23, 37, 38] It is thought that these deficiencies may be caused by many factors, such as gastrointestinal **(**GI) problems, dietary restrictions, and causes arising from the immune system.[39] These children were found to have deficiencies in cellular methylation and glutathione-mediated antioxidant defense in the metabolic process. It has been suggested that supplementation of vitamin B_{12}, which is a cofactor in the metabolism of methionine in transmethylation transsulfuration metabolism, may increase methylation and antioxidant capacities in these children.[40] In addition, oxidative stress in ASD has popularized the use of vitamins C and E.[41] In one study, 20 children with ASD aged 3-8 years were given vitamins B_6 and C for 3 months. Improvements in sleep problems and GI problems were seen in children.[42] In the study investigating the effects of vitamin A supplementation on the intestinal microbiota in children with ASD, plasma retinol levels and the rate of ASD-related *Bacteroidales* increased significantly at the end of six months. However, the relationship between vitamin A and ASD and microbiota has not been explained.[43] In a study conducted on a 14-year-old boy with ASD, vitamin B_{12} levels were found to be low and homocysteine levels high. The child was given 1000 µg of vitamin B_{12} supplementation for the first 5 days, then 1000 µg of vitamin B_{12} per month (as an injection once a month) for 8 weeks, and 500 mg of vitamin C and 400 mg of vitamin E daily. According to the results of the study, improvements in eye contact, touch, and walking and decreases in repetitive behaviors were seen in the children.[44] In another study, children with ASD were

given methylcobalamin by subcutaneous injection and folinic acid by oral route. The result of the study was an increase in cysteine and glutathione concentrations and an improvement in autistic behavior.[45] Although it is thought that the comorbidities and symptoms of ASD can be alleviated with vitamin supplements, more research is needed on this subject.[46]

Mineral

It has been shown in several studies that children with ASD have a higher prevalence of iron deficiency and iron deficiency anemia than other children.[47-49] Inadequate dietary iron intake and malabsorption are thought to cause iron deficiency.[50-52] Inadequate intake of iron by diet has been associated with frequent food selectivity in ASD.[53] Although it is thought that food selectivity leads to nutrient deficiency in ASD, the relationship between nutrient selectivity and nutrient deficiency has not been clearly determined.[54] A study on children with ASD showed that children with ASD were less likely to take in dairy products and, therefore calcium.[15] It has been suggested that the difference in copper and zinc levels in children with ASD may cause concentration difficulties, excessive mobility, and impulsivity. In one study, it was found that plasma erythrocyte and zinc levels of children with ASD were lower than in healthy children.[55] Zinc supplement is frequently seen in ASD.[56] According to a meta-analysis study, it was found that the plasma zinc level of the group with ASD was lower than the control group. However, no relationship was found between zinc levels and autism severity.[57] It has been suggested that zinc may be an important mineral in the treatment of ASD because metallothionein regulates gene expression and reduces heavy metal toxicity.[58] Mousain-Bosc, Roche, et al. examined the effects of magnesium and B6 supplementation in pervasive developmental disorders (PDD) and found that 70% of children with PDD aged 1-10 years improved social interaction, communication, abnormal/delayed functioning, and limited stereotyped behaviors.[59] Although vitamin and mineral supplementation is a recommended intervention for ASD, the number of studies on this subject is small.[60]

Probiotics

Probiotics are thought to contribute to the improvement of ASD-related problems by changing the levels of metabolites that may be harmful in children with ASD.[61] Another hypothesis that probiotics are thought to be beneficial is that healthy gut flora has an effect on the brain and behavior through the gut-brain barrier. However, the results of studies on this subject are contradictory. In a 2012 study of children with ASD, children were given supplementation containing *Lactobacillus acidophilus* for 2 months. It was found that the ability to focus on work and perform a task increased in the intervention group compared to the control group. However, no significant behavioral differences were found between the two groups.[25] Similar results were found in a placebo double-blind controlled study. Positive improvements in fecal microbiota, gut function, and behavior scores were found with the use of probiotics.[62] According to current studies, neurotransmitters and neuro immune responses in the microbiota-gut-brain axis may be targeted by probiotics, but the therapeutic effects of probiotics cannot be definitively explained. In addition, there is no definitive report on the improvement of ASD symptoms after probiotic treatment. Therefore, more studies on the use of probiotics in ASD are needed.[63]

Feeding Problems in ASD

Although studies show that the nutritional problems seen in ASD begin with the transition to complementary nutrition, the data on this subject is limited.[64] Although selective eating is common in both healthy children and children with developmental problems, some researchers say that selective eating may be a preliminary symptom of autism.[65] Since sensory integration problems are more common in children with ASD, this situation affects children's eating behaviors. Sensory sensitivity, such as consuming foods according to their colors and smelling foods, can be observed.[66] Compared to parents of healthy children, parents of children with ASD reported that their children had problematic eating behaviors more frequently.[15] It is stated that GIS complaints, which are frequently seen in ASD, can lead to nutritional problems, while nutritional problems can also lead to GIS complaints.[67, 68] Early recognition of nutritional problems and intervention is very important in terms of obtaining an effective result. Because eating disorders seen at an early age can affect cognitive, behavioral, and motor development and cause different behavioral problems.[69]

Symptoms of the gastrointestinal tract in ASD

According to a meta-analysis study conducted by Berry, Noval et al., it was found that children with autism had 4 times more GI complaints than children with normal development, diarrhea and constipation were three times more common, and abdominal pain

was twice as high.[70] Food selectivity in children with ASD may cause GI symptoms such as diarrhea, constipation, or exacerbation of symptoms. These problems increase in these children since simple carbohydrate foods such as sugary foods and snacks are preferred, and vegetables and fruits are not preferred much. Carbohydrate-heavy diets are thought to increase the osmotic load and cause diarrhea.[71] Nutritional management is important in the treatment of GI problems. However, there is no guidance on the nutrition of children with ASD. To develop a guideline that can be used in the clinic, it is necessary to bring together nutritionists who will be particularly interested in the nutrition of children with ASD.[70]

Special diets used in the medical nutrition therapy of ASD

Common special nutritional approaches in the therapy of ASD are gluten-free-casein-free diet, ketogenic diet, special carbohydrate diet, Feingold diet, Candida body ecology diet, eliminated allergy diet, and multivitamin, fatty acid, mineral, and probiotic supplements.[72]

Gluten-free-casein-free diet

The Gluten Free Casein Free (GFCF) diet is widely used to treat autism.[73] According to the "Opioid Excess Theory", there are enzyme defects associated with gluten and casein digestion in children with autism. As a result, it is assumed that intestinal permeability increases in children and the child is adversely affected. Opioids, which occur when foods containing gluten and casein cannot be fully digested, increase intestinal permeability, leak into the bloodstream and cross the blood-brain barrier. In addition, neurotransmission and endogenous opiate system may be affected and may cause impairment of central nervous system function.[74-77] This theory explains that gluten and casein-based peptides play a role in the etiology of autism and form the basis of GI system problems.[74] According to the theory, an increase in the level of opioid peptides in the urine can be considered a symptom of autism. Poor nutrition of these peptides can lower the level in urine and improve the behavioral symptoms seen in autism.[78] In the GFCF diet, all foods and beverages containing gluten and casein protein are excluded from the diet. Gluten is found in wheat, barley, oats, and rye, and casein is found in milk and dairy products.[79, 80] Although the GFCF diet is widely used, controlled studies demonstrating its effectiveness in the treatment of ASD are limited. A two-stage, 24-month-long single-blind randomized controlled trial of children with autism included 72 Danish children with autism aged 4-11 years. In the first stage, the children were divided into two groups; one group followed the GFCF diet and the other group did not. Participants were tested at baseline, at 8 and 12 months. According to the analyzes made in the 12th month, significant improvements were seen in behavior problems. In the other stage, participants were tested at 24 months. Although the group in the diet intervention plateaued in behavioral problems, it was seen that the improvement continued.[81] In the treatment of ASD, it has been stated that the diet can be applied if an allergy or intolerance to gluten and casein is diagnosed and there are positive improvements in the symptoms of autism with diet therapy.[82]

Ketogenic diets

Ketogenic therapy has been applied for years in the treatment of epilepsy. The ketogenic diet (KD), which is also a preferred method in the treatment of ASD, is restricted from carbohydrates and has a low protein and high-fat content. In classical KD, the ratio of fat to carbohydrate and protein in grams is 4:1. 90% of dietary calories come from fats.[83] Individuals on a ketogenic diet have changes in insulin and leptin levels, a decrease in serum glucose, and an increase in serum ketone levels and mitochondrial functions. The use of ketone bodies as an energy source has been shown to reduce metabolic disorders and symptoms in ASD.[84] 30 children aged 4-10 years were included in a pilot study, which was conducted prospectively in children with ASD. The children were given the John Radcliffe diet. The content of energy in this diet includes 30% medium-chain fatty acid, 30% fresh cream, 11% saturated fat, 10% protein, and 19% carbohydrate. 40% of the children who participated in the study could not tolerate or adapt to the diet. Positive changes in CARS scores were seen in children who completed the study.[85] Since the ketogenic diet contains limited amounts of carbohydrates, proteins, and other nutrients, it can adversely affect growth and cause weight loss. The application of this diet is thought-provoking because of the occurrence of eating disorders in children with ASD and their tendency to have low body weight. These symptoms can be reduced by adding thiamine, carnitine, and lipoic acid to the diet. However, it is recommended to evaluate the risks of dyslipidemia, beta-oxidation defect, acidosis, and mitochondrial and cardiovascular diseases that may occur as a side effect of the ketogenic diet.[84] Although the studies showing that autism symptoms improve with ketogenic diet application in children with ASD with epilepsy are

limited, it is thought to be promising in the treatment of these symptoms .[86]

Special carbohydrate diet

In treating irritable bowel syndrome and celiac disease, the Special Carbohydrate Diet (SCD) is applied to ensure the balance of bacteria in the intestine.[87]

In SCD, only monosaccharides are allowed from carbohydrates, and most disaccharides and polysaccharides are restricted.[88] Meat, eggs, homemade yogurt, vegetables, fruit oilseeds, and flours of oilseeds are included in the SCD diet.[87, 88] From cereals, wheat, oats, and rice, processed meats, canned vegetables and fruits, dried legumes, milk and dairy products, tuber vegetables (potato, sweet potato), curry, onion, and garlic powder are limited.[70] The studies in a four-year-old boy diagnosed with ASD and Fragile X Syndrome investigated SCD efficacy on GI issues. Improvement in GI symptoms was found.[89] More studies are needed on the effects of SCD on individuals .[90]

Feingold diet

Phenol is an organic compound, and occurs naturally in salicylates. It is also chemically produced from petroleum derivatives and used as a protective and coloring artificial food additive.[72] It has been observed that preservatives and colorants can cause hyperactivity in children. In addition, phenol sulfur transferase (PST) deficiency has been detected in individuals with ASD. Salicylates are a phenol subgroup that can be difficult to break down at times in individuals with ASD.[91] Dr. Feingold's hypothesis is that hyperactive symptoms decrease with the elimination of nutrient additives.[92] Therefore, it is recommended to eliminate foods containing protective, coloring, sweetening, flavoring Beta Hydroxy Acids (BHA), Butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) from the diet. Spices such as almonds, apricots, apples, strawberries, cucumbers, curries, oranges, honey, grapes, raisins, peaches, tomatoes, and peppers with common reactive salicylate content are also restricted.[72] A semi-experimental study was conducted in the city of Mansoura to investigate the effects of combining the Feingold diet with language education on language skills in children with ASD. However, no effect of diet on the development of language skills of children with ASD was observed.[93]

Candida body ecology diet (BED)

Candida albicans, a yeast-like fungus, can cause infection, especially in individuals with suppressed immune systems. The excessive increase of *Candida albicans* in the body has been associated with impaired concentration, hyperactive behaviors, and aggression in children with ASD. In addition, these problems can be seen together with headaches, stomach problems, gas pain, depression, and fatigue. Antifungal drugs, probiotic supplements, and sugar-poor diets are methods applied as preservatives.[77] BED is applied to prevent the excessive growth of candida, protect intestinal health, and maintain acid-base balance. This diet contains low-acid-forming foods, easily digestible foods without low sugar and starch content, and fermented foods. Pickled varieties from fermented foods, kefir, and yogurt made from non-animal milk are also included in the diet. In addition to being a gluten-free diet, BED does not contain rice, corn, and soy. Quinoa, millet, amaranth, and whole wheat can be consumed by soaking them neatly.[72]

Eliminated allergy diets

Children with ASD may have food sensitivity due to problems in the digestive and immune systems. Beneficial bacteria in the intestines react to carbohydrates and amino acids that cannot be digested. If a child is suspected of food allergy or intolerance, the necessary tests should be performed, or suspicious foods should be removed from the diet for two weeks, and when the same food is added to the diet again, it should be observed whether there are allergic symptoms. Since allergic foods are removed from the diet with the elimination diet, there may be improvements in the GI tract, behavior, and attention level. Foods excluded from the diet include milk, soy, wheat, fish, shellfish, eggs, nuts, and peanuts.[72]

Low FODMAP diet

FODMAP stands for "fermented oligo-di-monosaccharide and polyols." Carbohydrates in this group have high osmotic properties. The absorption of short-chain carbohydrates and polyols, which can be fermented at high levels, is poor. In this diet, these carbohydrates, which can ferment, are restricted. Malabsorption and sensitivity to FODMAP group saccharides may lead to GI symptoms. FODMAP group carbohydrates are fermented by bacteria in the intestine and cause gas formation. In case of malabsorption, the volume of water in the colon increases and causes diarrhea.[94] Foods that are forbidden by a low-FODMAP diet are removed and reintroduced into the diet.[95] Apples, pears, peaches, apricots, and dried fruits with a high fructose content; because of lactose-containing milk and dairy products; vegetables and fruits containing fructans such as artichokes, cauliflower, onions, okra, leeks,

broccoli, chickpeas, lentils, beans, wheat and rye , artificial sweeteners such as sorbitol and mannitol are restricted in this diet.[96,97] Although the FODMAP diet has been shown to provide significant reductions in GI complaints in inflammatory bowel diseases such as Irritable Bowel Syndrome (IBS), the data for its use in ASD are insufficient.[98]

CONCLUSION AND RECOMMENDATIONS

In conclusion, although the studies are promising, the evidence of diet practices reduce the severity of ASD is limited. Therefore, it is recommended to monitor children with ASD in terms of inadequate and unbalanced nutrition and to consider feeding problems and malnutrition in applying restrictive and special diets.

Authorship contribution statement

Consept and desing: MA, SÇ, ZB

Acquisition of data: MA, SÇ, ZB

Analysis and interpretation of data: MA, SÇ, ZB

Drafting of the manuscript: MA, SÇ, ZB

Critical revision of the manuscript for important intellectual content: MA, SÇ, ZB

Statistical analysis: -

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