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ORIGINAL ARTICLE

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Assessment of miR-1179 As a Potential Biomarker in Juvenile Myoclonic Epilepsy

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ABSTRACT

Objective: Juvenile myoclonic epilepsy (JME) is one of the most common childhood types of epilepsy and comprises 5-10% of all epilepsies. Altered expression levels of microRNAs (miRNAs) have been reported in epilepsy as in many diseases. As is known, miRNAs regulate gene expression post-transcriptionally and have potential as diagnostic biomarkers due to their stability in clinical samples. Herein, this study aimed to evaluate miR-1179 levels of JME patients and assess the potential of miR-1179 as a diagnostic biomarker.

Materials and Methods: Twenty patients and 20 healthy controls were recruited in this study and total RNA was extracted from peripheral blood samples of participants. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to calculate the relative expression level of miR-1179. Additionally, receiver operating characteristic (ROC) curve was conducted to evaluate the diagnostic value of miR-1179 in JME.

Results: Expression levels of miR-1179 were statistically significantly increased in patients with JME compared to healthy controls ($p<0.0001$). ROC analysis revealed that miR-1179 is a well diagnostic biomarker with an area under the curve (AUC) of 0.89.

Conclusion: miR-1179 may be considered a remarkable biomarker in the diagnosis of JME. The interaction between miR-1179 and its target *Calmodulin 1 (CALM1)* should be reinforced through functional studies. Further research in larger cohorts will help to enlighten the etiopathogenesis of JME.

Keywords: Juvenile myoclonic epilepsy, miR-1179, ROC curve, biomarker

INTRODUCTION

Epilepsy, which is estimated to affect over 50 million people worldwide, is a serious and chronic neurological disease that impacts public health. Epilepsy is a term that refers to an enduring predisposition to epileptic seizures which are a transient occurrence of symptoms caused by synchronous or abnormal excessive electrical functioning in the brain (1, 2). Juvenile myoclonic epilepsy (JME) is an idiopathic generalized epilepsy syndrome that affects 5-10% of individuals with epilepsy. Onset of JME is related to age, generally manifests between 12-18 years, with gender bias towards female (3). The genetic inheritance of JME is not entirely clarified, a multifactorial mechanism is assumed, however, several genes have been associated with JME (4). The heterogeneous and multifactorial nature of epilepsy leads to challenges in determining biomarkers for diagnosis and prognosis. However, determining biomarkers is crucial and would help to develop targeted therapies. In this context, there are several notable studies evaluating the biomarker potential of microRNAs (miRNAs) in epileptogenesis (2).

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miRNAs are small non-coding regulatory RNAs with their role in regulating post-transcriptional target gene expression through mRNA degradation or translational repression (5). miRNAs have a main role in various biological processes and are notable due to their potential in disease diagnostics and use as therapeutics. In particular, the stability of miRNAs in clinical samples has drawn attention to be used as disease biomarkers (6, 7). Altered expression levels of miRNAs have been associated with various conditions such as cardiovascular diseases, cancer, sepsis, and neurological diseases (8).

Herein, the expression levels of miR-1179 have been investigated in patients with JME. According to the miRTarBase database, *Calmodulin 1* (*CALM1*) is one of the target genes of miR-1179. It is estimated that hsa-miR-1179 may bind to the 3'UTR sequence of the *CALM1* gene at positions 1469–1491, 2032–2051, or 2378–2398 for miRNA-target interaction predicted by miRanda (9). According to the STRING database, the CALM1 protein and the CACNA1A protein interact which is validated by experimental determination, text mining, and co-expression data (Figure 1) (10). Previously, genetic changes in *CACNA1A* have been associated with the pathogenesis of JME (11).

In the context of this study, peripheral blood samples were collected from individuals diagnosed with JME and healthy controls, and the relative expression levels of miR-1179 were compared using the quantitative real-time polymerase chain reaction (qRT-PCR) method. To the best of this researcher's knowledge, miR-1179 has not been analyzed in any neurological disease yet. The study findings suggest that miR-1179 should be further analyzed with large-scale research to enlighten its role in the etiopathogenesis of JME.

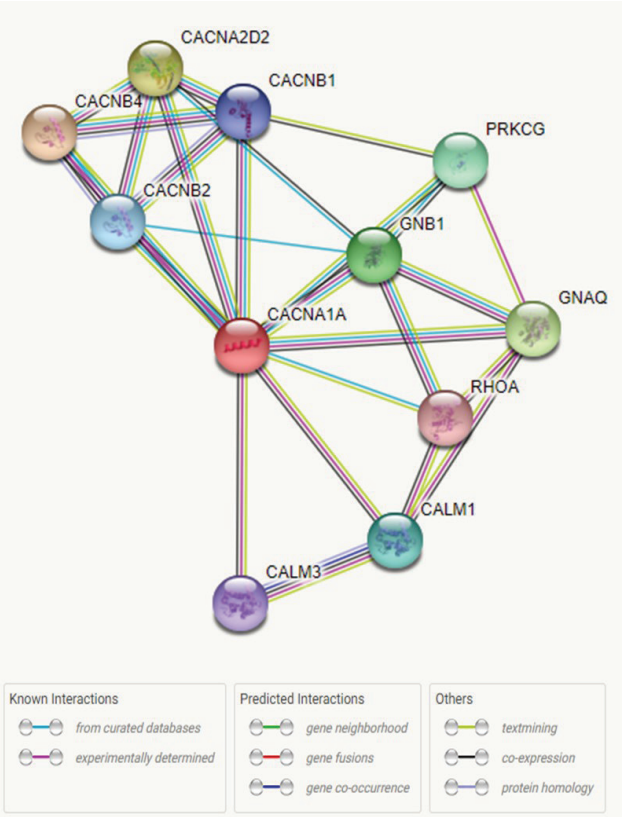

Figure 1. Interaction between CACNA1A and CALM1 proteins was visualized by the STRING database. Each color line indicates different interaction evidence.

MATERIALS AND METHODS

Patients Recruitment

This study protocol was approved by the ethics committee of Bezmialem Vakif University Faculty of Medicine (06.04.2021-E.11759), and written informed consent was obtained from all subjects. In the present study, 20 patients with JME (female:13, male:7) who were examined in Bezmialem Vakif University, Faculty of Medicine, Department of Neurology, and 20 healthy controls (female:12, male:8) without any neurological findings were recruited.

RNA Extraction and qRT-PCR

Five ml of peripheral blood samples was obtained in EDTA tubes from all participants (20 sample and 20 controls). Total RNAs were extracted using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) according to recommendations of the manufacturer. The quality and quantity of RNA were assessed via the Multiskan GO (Thermo Fisher Scientific, Boston, MA, USA). Two hundred fifty ng of the total RNA was converted to cDNA through a miRNA All-in-One cDNA Synthesis Kit (AbmGood, Vancouver, BC, Canada). The cDNAs were diluted 1:10 and used as templates for qRT-PCR. A qRT-PCR was performed on the Bio-Rad CFX96 Connect Real-Time PCR instrument (Bio-Rad Laboratories, Inc., California, USA) using BlasTaq 2X qPCR MasterMix (AbmGood), and data was obtained by Bio-Rad CFX Maestro software. The reaction conditions were as follows: 95˚C for 3 min, and 40 cycles of 95°C for 15 s, 60°C for 10 s, 72°C for 50 s. The miR-1179 forward primer (#MPH01026) and universal 3' miRNA reverse primer (#MPH00000) were purchased from AbmGood. A RNU6 was used as a reference gene. Relative miRNA expression levels were calculated by the $2^{-\Delta Ct}$ method (12).

Statistical Analyses

The GraphPad Prism 8.0 (GraphPad Software, Inc., CA, USA) was used to perform statistical analyses with a 95% confidence level and 0.05 significance level. Whether the data were distributed normally was tested with the Shapiro-Wilk test. When comparing the data with non-normal distribution between two independent groups the Mann-Whitney test was used. Additionally, to assess the diagnostic value of miRNAs, receiver operating characteristic (ROC) curves were generated, and area under the curve (AUC) values were calculated.

RESULTS

In the present study, the patients with JME comprise seven males and 13 females, and age-matched controls comprise eight males and 12 females. The averages of the cycle threshold (Ct) values obtained by the qRT-PCR study are

presented in Table 1. Accordingly, expression levels of miR-1179 in the samples obtained from peripheral blood were statistically significantly increased in patients compared to healthy controls ($p<0.0001$, Figure 2). The AUC value was determined as 0.89 at the 95% confidence level by a ROC curve analysis ($p<0.0001$, Figure 3).

Table 1. The average expression levels of miR-1179.

	miR-1179 Ct_{mean}	RNU6 Ct_{mean}	ΔCt	$2^{-\Delta Ct}$
Patients	34.34	28.34	6.00	0.02
Controls	33.56	26.31	7.25	0.01

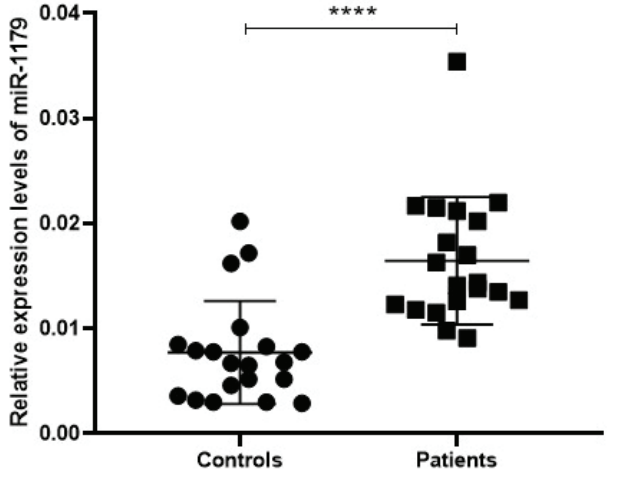

Figure 2. Relative expression levels of miR-1179 in patients and controls. **** indicates $p<0.0001$.

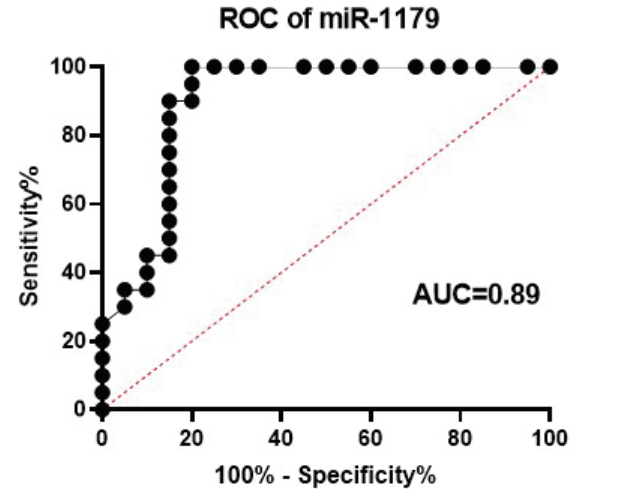

Figure 3. ROC curve analysis results reveal the diagnostic performance of miR-1179.

DISCUSSION

Epileptogenesis which includes many molecular and cellular processes is a complex process that has not been enlightened. Different epilepsy syndromes may have different etiology. Therefore, the determination of biomarkers associated with epilepsy is quite important to be used for diagnosis, prognosis, and treatment (13).

Applications of miRNA-based biomarkers have recently been an extensive and remarkable field of investigation. miRNAs are the most investigated subtypes of regulatory small non-coding RNAs. miRNAs are promising biomarkers thanks to their stability in extracellular areas, high stability in pre-clinical samples, and tissue specificity (14). Lots of research suggests that miRNAs have shown altered expression levels and/or dysfunction in various conditions with different types of cancer, cardiovascular disease, Parkinson's disease, epilepsy, multiple sclerosis, Alzheimer's disease, glioblastoma, and myasthenia gravis (8).

Studies published in PubMed from 2000 to 2017 to determine miRNA biomarkers in epilepsy patients were compiled by Yihong Ma et al., and according to this review, decreasing expression levels of miR-134 could be a diagnostic biomarker for epilepsy. miR-181 level downregulates in the acute stage of epilepsy, and this data supports that miRNA could be used to specify the disease phase. In addition, miR-15a-5p, miR-128, miR-199a, miR-124, and miR-194-5p could be candidate diagnostic biomarkers in patients with epilepsy (15). In a recent study by Ferreira et al., serum levels of miR-155 and miR-146a were found up-regulated in patients with genetic generalized epilepsy compared to controls, therefore, these miRNAs were suggested as diagnostic biomarkers (13). Another study indicated that serum levels of miR-106b and miR-146a were statistically significantly increased in patients with focal and generalized epilepsy (16).

Taken together, miRNAs should be considered as remarkable biomarkers in the diagnosis and prognosis of epilepsy. Therefore, this study aimed to assess the diagnostic biomarker performance of miR-1179 in JME. To the best of this researcher's knowledge, the expression level of miR-1179 has not been evaluated in any neurological disorders to date. According to study results, miR-1179 was significantly upregulated in JME patients compared to healthy controls.

As known, ROC curve analysis is used as an efficient graphical tool to evaluate the diagnostic significance of biomarkers by calculating the sensitivity and the specificity of interested miRNA (17). In the present study, the AUC value was calculated through ROC curve analysis to evaluate miR-1179 as a diagnostic biomarker. Accordingly, this study suggests miR-1179 as a useful diagnostic biomarker in JME.

According to miRTarBase, hsa-miR-1179 targets *CALM1* mRNA from the 3'UTR (9). *CALM1* (ENSG00000198668) encodes Calmodulin-1 protein (Uniprot ID: P0DP23). Calcium (Ca^{2+}) flux

across cell membranes play a key role in the cellular responses while calmodulin (CaM) perceives Ca^{2+} concentration changes. CaM detects such alteration, and it relays this information to interaction partners. The accurate Ca^{2+} signaling is driven by three independent calmodulin-encoding genes (*CALM1-3*) in the genome and these three genes encode entirely the same functional CaM protein (18). CaM regulation of Ca^{2+} channels is the main point to Ca^{2+} signaling (19). The *CACNA1A* encodes a subunit of the voltage gated Ca^{2+} channel. Further, voltage dependent Ca^{2+} channels are involved in various biological processes such as releasing a neurotransmitter or hormone, muscle contraction, gene expression, and pathogenic changes in *CACNA1A* that have been associated with numerous diseases (MIM: 601011).

In 2001, Chioza et al. showed that *CACNA1A* was directly related to the etiopathogenesis of the idiopathic generalized epilepsy group which comprises JME (20). According to the STRING, which is a database that reliably presents protein-protein interactions based on both experimental and literature research data, the CACNA1A and CALM1 proteins are directly related (10). Consequently, it may be speculated that *CALM1* contributes to the etiology of JME. Based on this information, this study suggests that miR-1179 is involved in the pathogenesis of JME by targeting *CALM1* mRNA.

CONCLUSION

Considering calcium channelopathies in epilepsy, the low expression of *CALM1* due to increased expression of miR-1179 could be a role in the etiopathogenesis of JME. Additionally, this study emphasizes that miR-1179 could be a useful diagnostic biomarker in JME. To this researcher's knowledge, the present study may be the first in terms of evaluating miR-1179 in any neurological disease. Further analysis and functional studies in larger cohorts may enlighten the effect of miR-1179 on the pathogenesis of JME and could reveal its diagnostic value accurately.

Ethics Committee Approval: This study was approved by the ethics committee of Bezmialem Vakif University Faculty of Medicine (06.04.2021-E.11759).

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Author Contributions: Conception/Design of Study - S.S., C.T., A.B., F.U., E.Y.; Data Acquisition - S.S., C.T., A.B., F.U., E.Y.; Data Analysis/Interpretation - S.S., E.Y.; Drafting Manuscript - S.S.; Critical Revision of Manuscript - E.Y.; Final Approval and Accountability - S.S., C.T., A.B., F.U., E.Y.

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

REFERENCES

1. Moshe SL, Perucca E, Ryvlin P, Tomson T. Epilepsy: new advances. Lancet 2015; 385(9971): 884-98. [CrossRef]
2. Pitkanen A, Loscher W, Vezzani A, Becker AJ, Simonato M, Lukasiuk K, et al. Advances in the development of biomarkers for epilepsy. Lancet Neurol 2016; 15(8): 843-56. [CrossRef]
3. Pietrafusa N, La Neve A, de Palma L, Boero G, Luisi C, Vigevano F, et al. Juvenile myoclonic epilepsy: Long-term prognosis and risk factors. Brain Dev 2021; 43(6): 688-97. [CrossRef]
4. Amrutkar C, Riel-Romero RM. Juvenile Myoclonic Epilepsy. 2021 Aug 11. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022.
5. Vishnoi A, Rani S. MiRNA Biogenesis and Regulation of Diseases: An Overview. Methods Mol Biol 2017; 1509: 1-10. [CrossRef]
6. Hydbring P, Badalian-Very G. Clinical applications of microRNAs. F1000Res 2013; 2: 136. [CrossRef]
7. Süsgün S, Karacan İ, Yücesan E. Optimization of One Step Reverse Transcription Quantitative PCR Method for miRNA Expression Analyses. Experimed 2021; 11(2): 113-9. [CrossRef]
8. Condrat CE, Thompson DC, Barbu MG, Bugnar OL, Boboc A, Cretoiu D, et al. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. Cells 2020; 9(2). [CrossRef]
9. Huang HY, Lin YC, Li J, Huang KY, Shrestha S, Hong HC, et al. miRTarBase 2020: updates to the experimentally validated microRNA-target interaction database. Nucleic Acids Res 2020; 48(D1): D148-D54. [CrossRef]
10. Szklarczyk D, Gable AL, Nastou KC, Lyon D, Kirsch R, Pyysalo S, et al. The STRING database in 2021: customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. Nucleic Acids Res 2021; 49(D1): D605-D12. [CrossRef]
11. Dziadkowiak E, Chojdak-Lukasiewicz J, Olejniczak P, Paradowski B. Regulation of microRNA Expression in Sleep Disorders in Patients with Epilepsy. Int J Mol Sci 2021; 22(14). [CrossRef]
12. Riedel G, Rudrich U, Fekete-Drimusz N, Manns MP, Vondran FW, Bock M. An extended DeltaCT-method facilitating normalisation with multiple reference genes suited for quantitative RT-PCR analyses of human hepatocyte-like cells. PLoS One 2014; 9(3): e93031. [CrossRef]
13. Martins-Ferreira R, Chaves J, Carvalho C, Bettencourt A, Chorao R, Freitas J, et al. Circulating microRNAs as potential biomarkers for genetic generalized epilepsies: a three microRNA panel. Eur J Neurol 2020; 27(4): 660-6. [CrossRef]
14. Krauskopf J, Verheijen M, Kleinjans JC, de Kok TM, Caiment F. Development and regulatory application of microRNA biomarkers. Biomark Med 2015; 9(11): 1137-51. [CrossRef]
15. Ma Y. The Challenge of microRNA as a Biomarker of Epilepsy. Curr Neuropharmacol 2018; 16(1): 37-42. [CrossRef]
16. An N, Zhao W, Liu Y, Yang X, Chen P. Elevated serum miR-106b and miR-146a in patients with focal and generalized epilepsy. Epilepsy Res 2016; 127: 311-6. [CrossRef]
17. Hsu MJ, Chang YC, Hsueh HM. Biomarker selection for medical diagnosis using the partial area under the ROC curve. BMC Res Notes 2014; 7: 25. [CrossRef]
18. Jensen HH, Brohus M, Nyegaard M, Overgaard MT. Human Calmodulin Mutations. Front Mol Neurosci 2018; 11: 396. [CrossRef]

19. Mori MX, Vander Kooi CW, Leahy DJ, Yue DT. Crystal structure of the CaV2 IQ domain in complex with Ca2+/calmodulin: high-resolution mechanistic implications for channel regulation by Ca2+. Structure 2008; 16(4): 607-20. [CrossRef]

20. Chioza B, Wilkie H, Nashef L, Blower J, McCormick D, Sham P, et al. Association between the alpha(1a) calcium channel gene CACNA1A and idiopathic generalized epilepsy. Neurology 2001; 56(9): 1245-6. [CrossRef]

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ORIGINAL ARTICLE

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Cytotoxic and Apoptotic Effects of Carmofur and Vitamin C Combination on HCT116 Colon Cancer Cells

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ABSTRACT

Objective: In recent years, especially combined treatment options that will reduce the side effects of drugs have attracted attention. Carmofur is a new potent agent being investigated in the treatment of cancer. This study aimed to investigate the cytotoxic and apoptotic effects of carmofur+vitamin C combination in colon cancer cells.

Materials and Methods: Carmofur in the range of 7.8-250 µM, vitamin C in 7.8 µM-2mM and carmofur+vitamin C in different concentrations were evaluated on HCT116 cells by MTT test. In addition, the cell death pathway was determined by an apoptosis-necrosis assay.

Results: The IC_{50} value of carmofur in HCT116 cells was 8 µM, vitamin C was not effective at low doses, and the IC_{50} value was 2.2 mM. When carmofur+vitamin C were applied to HCT116 cells, 4 µM carmofur and 125 µM vitamin C together inhibited half of the cells (IC_{50}), while 4 µM carmofur and 2 mM vitamin C inhibited half of the cells similarly.

Conclusion: Vitamin C doubled the anticancer effect of carmofur. As a result, it was observed that vitamin C supplementation significantly increased cell death in anticancer drug administration. In addition, it was determined that the addition of vitamin C significantly increased the apoptotic activity.

Keywords: Carmofur, vitamin C, combined therapy, colon cancer, HCT116

INTRODUCTION

Cancer is the second cause of death in the world and an important health problem. Colon cancer, which is one of the most common malignant diseases in humans, is the third most common cause of cancer mediated mortality in the most developed countries and worldwide (1). Despite the numerous approaches to prevent and treat cancer, for instance, chemotherapy, surgery and radiotherapy, and some advances in cancer treatment, there is still a necessity to improve effective, leading-edge cancer treatments (2, 3). However, the widespread side effects and reactions of the drugs limit the effectiveness of current cancer treatments (4). Therefore, it is necessary to find new approaches and find novel and effective drugs to reduce side effects in cancer treatment (5). In recent years, one of the drugs researched in cancer treatment is carmofur, which is a ceramidase inhibitor and a new potent candidate in cancer therapy. Carmofur, a masked form of 5-Fluorouracil (FU), is a pyrimidine analog used as an antineoplastic agent (6). Unlike 5-FU, carmofur is destroyed by the extrahepatic route as well as the hepatic route and its level in the blood may increase to higher rates (7). Carmofur has also been used as adjuvant chemotherapy for breast and colorectal cancer in some countries (7, 8). In addition, it is a very strong acid ceramidase (AC) inhibitor and causes ceramide accumulation in cancer cells. Ceramide acts as a messenger that activates apoptosis and cell differentiation (9, 10). In addition, some

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researchers reported that carmofur was more effective in targeting 5-FU resistant cells (11). However, the exact mechanism of carmofur on cancers is not known.

Vitamin C is an essential nutrient that our body needs for normal function and it has anticancer and antioxidant activities. Although vitamin C cannot be synthesized by the body, it is plentiful in many natural sources and can be taken as a dietary supplement. Moreover, it scavenges reactive oxygen species (ROS) and induces cytotoxicity against tumor cells, prevents glucose metabolism, acts as an epigenetic regulator, and plays an important role in preventing cancer development by many mechanisms (4). In *in vitro* studies, it has been observed that high concentrations of vitamin C inhibit cell migration and angiogenesis (12). Accordingly, vitamin C can inhibit the metastasis and proliferation of tumor cells. In addition, vitamin C is not significantly toxic to normal cells (13) and is low-cost and readily available, so it may be an excellent candidate to improve an effective anticancer agent.

Numerous approaches and materials are being researched for dealing with cancer. In this context, carmofur, an anticancer drug whose efficacy is not fully known, has recently emerged as a potential candidate. In addition, in order to reduce the side effects of cancer drugs and increase their therapeutic effect, the combination of natural compounds such as vitamin C is being investigated as a treatment option.

This study focuses on the importance of vitamin C in reducing the side effects while increasing the cytotoxic effect of anti-cancer drugs. The present study aimed to develop a new, more effective possible treatment strategy, besides reducing the side effects of carmofur in colon cancer, and examined the effect of the combination of vitamin C with carmofur on colon cancer cells. In the light of this information, in this study, the cytotoxic effect and death pathway of carmofur and vitamin C in HCT116 colon cancer cells, separately and combined, were investigated.

MATERIALS AND METHODS

HCT116 colon cancer cell lines obtained from ATCC were used in the present study. Dulbecco's-Modified-Eagle-Medium (DMEM; Sigma, D2902), $NaHCO_3$ (Sigma, S5761), penicillin-streptomycin (10,000 U/mL, 15140148), Fetal Bovine Serum (FBS, Sigma F4135) was used to prepare cell media. Cells were removed with trypsin (Sigma, T9935). MTT (Sigma, M2128) and Dimethyl sulfoxide (DMSO; Merck, 102952) were used for cytotoxicity determination. Cancer cells were treated with vitamin C (Sigma, A4403) and carmofur (Glentham, GP5925). Annexin V-FITC apoptosis Kit (Beckman Coulter, IM2375) was used for the determination of apoptosis.

Cell Culture Studies

HCT116 cells were cultured at 37°C in an atmosphere of 95% O_2, 5% CO_2, DMEM containing 100.000 U/L penicillin, 100.000 g/L streptomycin, 10% FBS was used as the medium. The medium of the cells was refreshed at least twice a week. Cells were passaged when they were 70-90% confluent.

Evaluation of Cell Viability by MTT Test

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) is a quantitative, colorimetric method which is used to quantitate cytotoxic activity based-on *in vitro* metabolism viability (14). In this study, stock solutions of the drugs were prepared for carmofur and vitamin C in dimethylsulfoxide (DMSO) (with DMSO ratio below 1%). Carmofur and vitamin C were then diluted to different concentrations with DMEM and incubated with HCT116 colon cancer cells for 72 hours in a 5% CO_2 incubator. 96-well culture plates were used and 90 µl of the cells 10^5 per milliliter were taken into wells. Then, carmofur was added in the range of 7.8-250 µM, vitamin C in the range of 7.8 µM-2 mM, and carmofur+vitamin C combined in different concentrations and added 10 µl separately to wells on the plate and incubated for 72 hours. After incubation, MTT (5 mg/ml, PBS) was added and incubated for 3 hours, and the absorbance was measured at 570 nm in an ELISA plate reader. According to dose/response curves, half-maximal inhibitory concentrations (IC_{50}) of the compounds were determined. Each test was repeated at least 3 times.

Determination of Apoptosis/Necrosis

In HCT116 cells, the rates of viable, apoptotic/necrotic cells were evaluated via the annexin V-FITC/propidium iodide. HCT116 cells were cultured in a 6-well plate as 3×10^5 cells per well with IC_{50} doses of the compounds. Evaluation of apoptosis and necrosis was performed at IC_{50} doses and for 72 hours to be compatible with MTT tests: Carmofur 8 µM, vitamin C 2.2 mM, carmofur (4 µM) + vitamin C (250 µM), carmofur (4 µM) + vitamin C (2 mM) concentrations were prepared for combination treatments. These concentrations were added to each well and incubated for 72 h. The cells were centrifuged for 10 minutes. The cell pellet was washed with 1 mL of PBS and centrifuged again. This procedure was repeated 3 times. Then, 100 µL of the cell pellet was stained by 5 µL annexin V-FITC and 2.5 µL propidium iodide. After 15 minutes, cells were measured on a Beckman Coulter flow cytometer, showing viable, early apoptosis (Annexin positive/PI negative), and late apoptosis (both Annexin/PI-positive) cells. The results were evaluated with the Kaluza analysis program.

Statistical Analysis

Data were first evaluated for normality via Shapiro-Wilk test. Samples were compared with one-way analysis of the variance (ANOVA) test, and Tukey, as a post-hoc-test, was utilized. $p<0.05$ was considered as statistical significance level and analyses were evaluated with GraphPad software.

RESULTS

Cytotoxic Effects of Carmofur and Vitamin C on HCT116 Cells

HCT116 colon cancer cells were incubated with carmofur alone, vitamin C alone, and combined concentrations of the two compounds. When carmofur was applied to colon cancer cells at 7.8, 15.6, 31.5, 62, 125 and 250 µM concentrations, the cell viability was 51%, 48%, 42%, 32%, 35%, and 26%, respec-

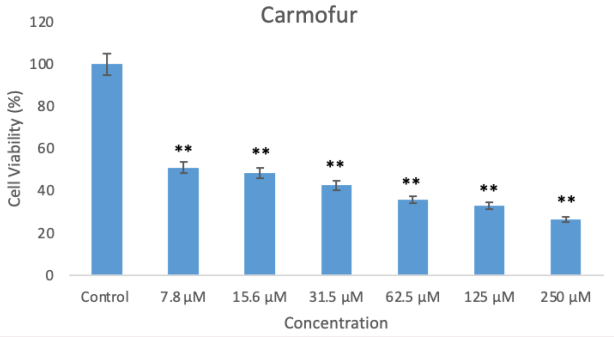

Figure 1. Cell viability effect of different concentrations of carmofur on HCT116 colon cancer cells. Cell viability tests were evaluated by MTT test after 72 hours of incubation. Experiments were repeated at least 3 times.

Statistical significance is shown with **p<0.01 compared to the untreated control group.

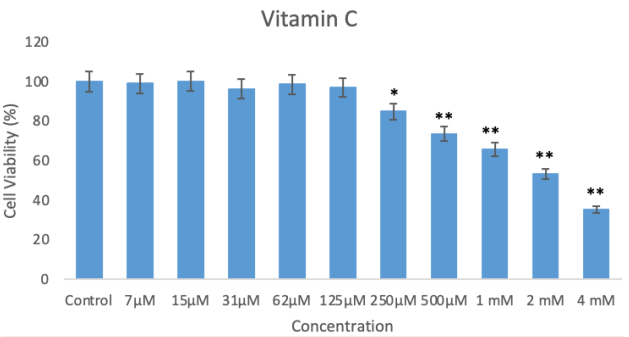

Figure 2. Cell viability effect of different concentrations of vitamin C on HCT116 colon cancer cell lines. Cell viability tests were evaluated by MTT test after 72 hours of incubation.

Statistical significance is shown by *p<0.05 and **p<0.01 compared to the untreated control group.

tively. When vitamin C is applied at 7.8, 15.6, 31.5, 62, 125 and 250, 500 µM, 1 mM, 2 mM and 4 mM concentrations, the viability of colon cancer cells was determined as 98%, 99%, 96%, 98%, 96%, 94%, 84%, 73%, 64%, 53% and 35% respectively (Figure 1, Figure 2).

It was observed that low-doses of vitamin C alone were not effective in cancer cells. The IC_{50} value of carmofur alone on colon cancer cells was determined at 8 µM, and the IC_{50} value of vitamin C alone was 2.2 mM. When carmofur and vitamin C were applied as a combination, 4 µM carmofur+125 µM vitamin C inhibited half of the cells (IC_{50}), while 4 µM carmofur+2 mM vitamin C had a similar effect (Figure 1, Figure 2, Figure 3, Figure 4).

Combined Cytotoxic Effects of Carmofur and Vitamin C on HCT116 cells

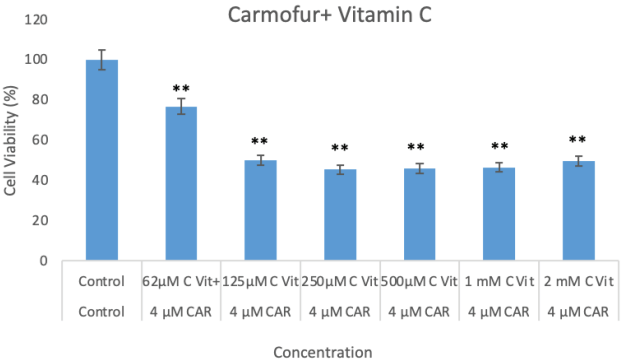

Figure 3. Cell viability effect of 4 µM carmofur concentration and different concentrations of vitamin C combination on HCT116 cells. Cell viability tests were evaluated by MTT test after 72 hours of incubation.

Statistical significance is shown with **p<0.01 compared to the drug-free control group. CAR: Carmofur.

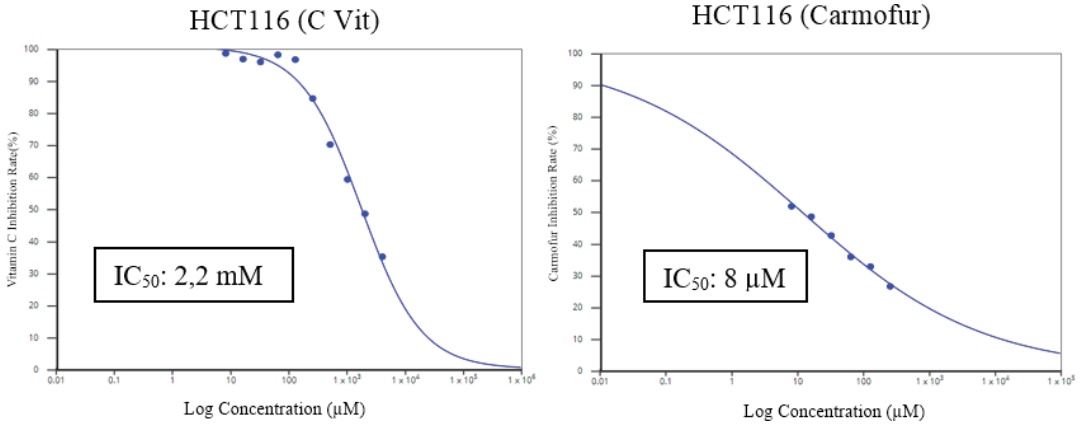

Figure 4. IC_{50} values of vitamin C and carmofur on HCT116 cells. Calculations were performed with "AAT bioquest IC_{50} calculator" program.

IC_{50}: Concentration that inhibits 50% of the cells.

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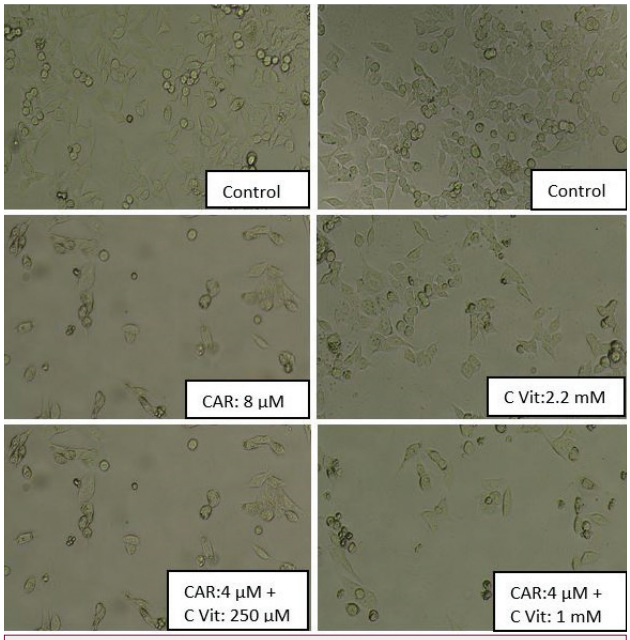

Figure 5. Morphological changes on HCT116 cells when carmofur and vitamin C were administered alone or together (Light microscope: 200X) (CAR: Carmofur).

Figure 5 shows the morphological changes in the untreated control group and treated HCT116 cells. It is observed that the cells of the untreated control group are fusiform, connected to each other, and adhered to the surface. There was a remarkable decrease in the number of cells in the groups where carmofur and vitamin C were administered together or alone. Shrinkage and rounding are observed in some cells, and the decrease in cell number and intercellular connections is noteworthy. In figure 5, images of the treated groups with approximate IC_{50} values are given. According to the findings obtained in this study, morphological changes are compatible with cytotoxicity and apoptosis tests.

Evaluation of Apoptotic-Necrotic Effects by Flow Cytometry Analysis on HCT116 Cells

Figure 6 shows the apoptotic and necrotic effects of carmofur and vitamin C alone or combined in HCT116 cancer cells. To see cell death in the apoptosis-necrosis assay, concentrations that inhibited about half of the cells (IC_{50}) were studied. Apoptosis-necrosis findings were consistent with cytotoxicity findings (Figure 6).

In addition, when we evaluated the apoptotic effect of carmofur alone at the IC_{50} concentration, 51% of the cells were alive, while 11% were under early apoptosis, 23% late apoptosis, and

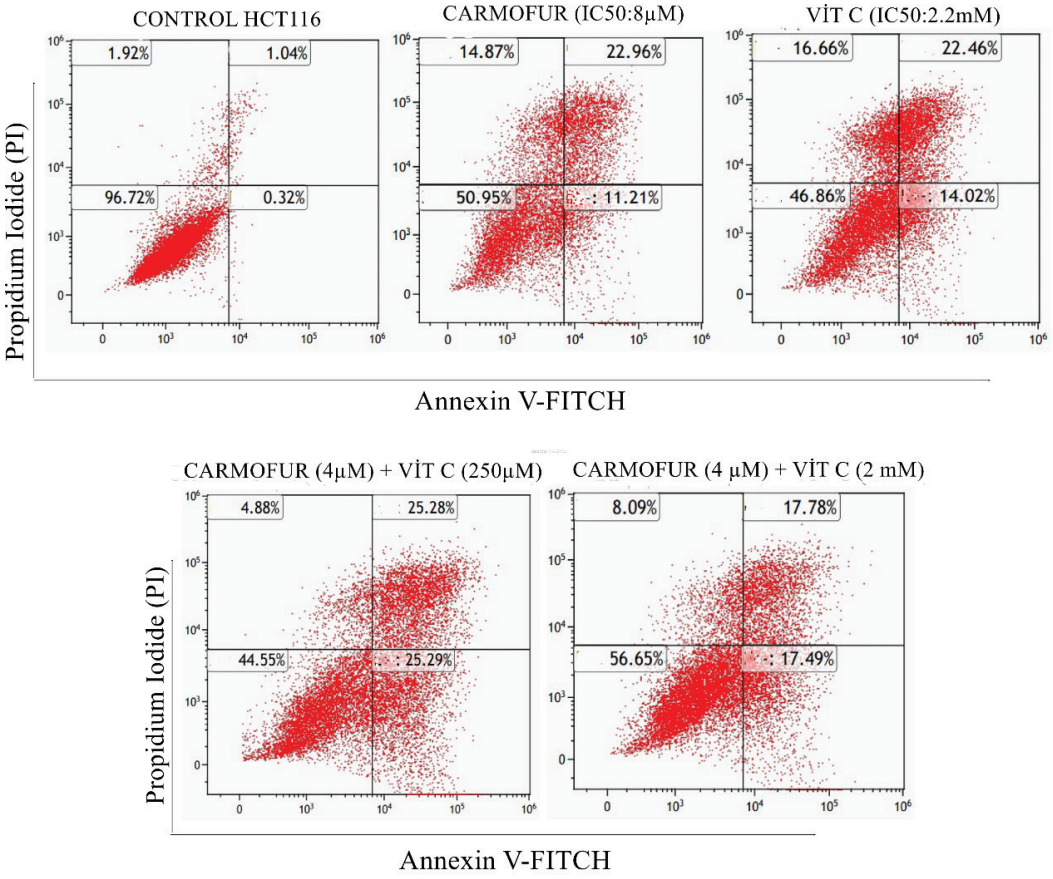

Figure 6. Flow cytometry analyses of HCT116 cells after incubations with C armofur and vitamin C alone and combined.

Table 1. Apoptotic necrotic rates (%) of carmofur alone, vitamin C alone, and carmofur+vitamin C combination on HCT116 colon cancer cells.

	Live (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis/Dead (%)
Control	96.72	0.32	1.04	1.92
Carmofur	50.95	11.21	22.96	14.87
Vitamin C	46.86	14.02	22.56	16.66
Carmofur (4 µM) + vitamin C (250 µM)	44.55	25.29	25.28	4.88
Carmofur (4 µM) + vitamin C (2 mM)	56.65	17.49	17.78	8.09

14% necrosis. When we evaluated the apoptotic effect of vitamin C alone at the IC_{50} concentration, 47% of the cells were alive, while 14% had early apoptosis, 22% had late apoptosis, and 16% had necrosis. Moreover, as a result of the combination of carmofur with low dose vitamin C (250 µM), it caused 45% cell viability, 25% early apoptosis, 25% late apoptosis, 4% necrosis, while vitamin C is used in high doses (2.2 mM) combined, 56% viable cells, 17% early apoptosis, 17% late apoptosis, 8% necrosis rate were observed (Figure 6, Table 1). According to these findings, when carmofur was used in combination with vitamin C, it was determined that apoptosis on cancer cells increased significantly.

DISCUSSION

In this study, the cytotoxic effects of carmofur and vitamin C at different concentrations on HCT116 colon cancer cells were evaluated. Carmofur, an acid ceramidase inhibitor (9), was quite effective in these cells, and the IC_{50} value was found to be 8 µM. For vitamin C, µM and mM concentrations were studied. Vitamin C alone did not show significant cytotoxicity in colon cancer cells at doses of 250 µM and below. The IC_{50} value obtained by the application of vitamin C alone, which is the concentration that inhibits 50% of the cells, was determined to be 2.2 mM (Figure 1, Figure 2). In addition, when 4 µM, which is half of the IC_{50} concentration of carmofur, and different concentrations of Vitamin C (in the range of 62 µM- 2 mM) are combined, it is observed that especially the concentrations of 125 µM vitamin C and above significantly increase cytotoxicity **(**Figure 3**).** Half of the HCT116 cells were inhibited (IC_{50}) when 4 µM carmofur (half the IC_{50} value) was co-administered with 125 µM vitamin C. In other words, with the support of vitamin C, the concentration of the chemotherapy drug decreased by half and showed the same effect. Vitamin C and carmofur showed a synergistic effect. Interestingly, similar cytotoxicity was detected when the vitamin C concentration was increased up to 2mM in combination treatment with carmofur.

Most interestingly, while vitamin C alone shows toxicity at high doses such as 2 mM, when used in combination with carmofur anticancer drug, it reaches the highest cytotoxicity at 125 µM concentrations, and there is no significant difference in combination between high doses such as 1mM and 2mM. While 8 µM carmofur had the IC_{50} value, 4 µM carmofur + 125 µM vitamin C shows the same effect. Similar to this study, Ghavami et al. reported that when they used vitamin C and cisplatin in combination, the addition of vitamin C increased cell death in gastric cancer cells (15). In another study, Lee et al. evaluated the combined effect of different anticancer drugs such as tamoxifen, fulvestrant, trastuzumab, and vitamin C on MCF-7, and MDA-MB-231 breast cancer cells. It has been reported that the combined use of vitamin C and anticancer drugs provides a therapeutic advantage in breast cancer cells (16).

The development of effective pro-apoptotic agents with drug discovery studies and understanding the mechanism are of great importance for cancer treatment (17). In this study, it is clearly seen that combined treatment with vitamin C triggers apoptotic activity. When we evaluate the apoptotic effect, when carmofur and vitamin C are applied alone, it is seen that some necrosis is triggered along with apoptosis **(**Figure 6, Table 1**).** When low dose (250 µM) vitamin C is applied combined with carmofur, it is seen that apoptotic effect is increased significantly (50%) and necrosis is decreased (4%). In addition to stimulation of apoptosis, vitamin C alone at a high dose (2.2 mM) also caused significant necrosis. However, when carmofur + vitamin C was combined, especially with low-dose vitamin C (250 µM), it is noteworthy that apoptosis increased significantly. According to these results, the addition of vitamin C in anticancer drug use induces apoptosis in cell death. Although there are some studies investigating the anticancer effect of carmofur alone, these are very limited, moreover, there is no study based on the use of carmofur together with vitamin C in the literature. In a study by Çömlekçi et al., the IC_{50} value of carmofur was 16 µM in A549 lung cancer cells (18), while it was reported in the range of 4.6-50 µM in pediatric brain tumors in another study (19). The cytotoxic activity found in this study is also consistent with other studies.

The high level of acid ceramidase in cancer cells increases resistance to treatment and complicates treatment (10). Carmofur is a very strong acid ceramidase inhibitor and can reduce

drug resistance, which is the biggest problem in treatment, by inhibition of acid ceramidase, especially in treatment-resistant cancers such as colon cancer (20). For this reason, in this study, it was shown that carmofur has a very strong anticancer effect on HCT116 cells, which are highly resistant to treatment, and this effect can be further enhanced with vitamin C. When we compared the effect of other anticancer drugs in many studies conducted to date, we showed that carmofur can be a powerful anticancer drug for colon cancer and this effect is multiplied by vitamin C.

Vitamin C and its derivatives are compounds that need further investigation with their potential to aid treatment. Vitamin C and its derivatives can offer important therapeutic options in cancer treatment with their advantages such as easy availability, low toxicity and low cost. However, further studies are needed on the dose and clinical benefits of vitamin C in cancer treatment.

CONCLUSION

This study demonstrates that co-administration of vitamin C and carmofur could be developed in the future as an innovative, effective therapeutic strategy for colon cancer patients. The combination of carmofur with vitamin C significantly increased cytotoxicity and apoptotic activity in colon cancer cells.

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Conflict of Interest: The author has no conflict of interest to declare.

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REFERENCES

1. Razali FN, Sinniah SK, Hussin H, Abidin NZ, Shuib AS. Tumor suppression effect of Solanum nigrum polysaccharide fraction on Breast cancer via immunomodulation. Int J Biol Macromol 2016; 92: 185-93. [CrossRef]
2. Sharma PC, Bansal KK, Sharma A, Sharma D, Deep A. Thiazole-containing compounds as therapeutic targets for cancer therapy. Eur J Med Chem 2020; 188: 112016. [CrossRef]
3. Sharma PC, Sharma D, Sharma A, Bhagat M, Ola M, Thakur VK, et al. Recent advances in microbial toxin-related strategies to combat cancer. In Seminars in Cancer Biology. Academic Press 2021. [CrossRef]
4. Reang J, Sharma PC, Thakur VK, Majeed J. Understanding the therapeutic potential of ascorbic acid in the battle to overcome cancer. Biomolecules 2021; 11(8): 1130. [CrossRef]
5. Miao Y, Xiao B, Jiang Z, Guo Y, Mao F, Zhao J. Growth inhibition and cell-cycle arrest of human gastric cancer cells by Lycium barbarum polysaccharide. Med Oncol 2010; 27(3):785-90. [CrossRef]
6. Zhang P, Liu S. Solvent Effects on the UV Absorption Spectrum of Carmofur. Detection 2016; 4: 25-31. [CrossRef]
7. Ken M, Masae K. Postoperative adjuvant use of carmofur for early breast cancer. Osaka City Med J 2003; 49(2):77-83.
8. Nakamura T, Ohno M, Tabuchi Y, Kamigaki T, Fujii H, Yamagishi H, et al. Optimal duration of oral adjuvant chemotherapy with Carmofur in the colorectal cancer patients: the Kansai Carmofur Study Group trial III. Int J Oncol 2001; 19(2): 29198. [CrossRef]
9. Realini N, Solorzano C, Pagliuca C, Pizzirani D, Armirotti A, Luciani R, et al. Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. Sci Rep 2013; 3: 1035. [CrossRef]
10. Clifford RE, Govindarajah N, Bowden D, Sutton P, Glenn M, Darvish-Damavandi M, et al. Targeting Acid Ceramidase to Improve the Radiosensitivity of Rectal Cancer. Cells 2020; 9(12): 2693. [CrossRef]
11. Maehara Y, Kusumoto H, Anal H, Kusumoto T, Hiramoto Y, Sugimachi K. 1-Hexylcarbamoyl-5-fluorouracil is more cytostatic than 5-fluorouracil against human tumors in vitro. Eur J Cancer Clin Oncol 1987; 23(10): 1511-5. [CrossRef]
12. Mikirova NA, Ichim TE, Riordan NH. Anti-angiogenic effect of high doses of ascorbic acid. J Transl Med 2008; 6(1): 1-10. [CrossRef]
13. Lee WJ. The prospects of vitamin C in cancer therapy. Immune Netw 2009; 9(5): 147-52. [CrossRef]
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983; 65(1-2): 55-63. [CrossRef]
15. Ghavami G, Sardari S. Synergistic Effect of Vitamin C with Cisplatin for Inhibiting Proliferation of Gastric Cancer Cells. Iran Biomed J. 2020; 24(2): 119-27. [CrossRef]
16. Lee SJ, Jeong JH, Lee IH, Lee J, Jung JH, Park HY, et al. Effect of high-dose vitamin C combined with anti-cancer treatment on breast cancer cells. Anticancer Res 2019; 39(2): 751-8. [CrossRef]
17. Carneiro BA, El-Deiry WS. Targeting apoptosis in cancer therapy. Nat Rev Clin Oncol 2020; 17(7): 395-417. [CrossRef]
18. Çömlekçi E, Kutlu HM, Sezer CV. Toward stimulating apoptosis in human lung adenocarcinoma cells by novel nano-carmofur compound treatment. Anti-cancer drugs 2021; 32(6): 657-63. [CrossRef]
19. Doan NB, Nguyen HS, Montoure A, Al-Gizawiy MM, Mueller WM, Kurpad S, et al. Acid ceramidase is a novel drug target for pediatric brain tumors. Oncotarget 2017; 8(15): 24753. [CrossRef]
20. Dementiev A, Joachimiak A, Nguyen H, Gorelik A, Illes K, Shabani S, et al. Molecular mechanism of inhibition of acid ceramidase by carmofur. J Med Chem 2018; 62(2): 987-92. [CrossRef]

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ORIGINAL ARTICLE

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Synthesis of Natural Salicylic Acid as a Cosmetic Ingredient Using Green Chemistry Methods

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ABSTRACT

Objective: Salicylic acid (SA) is a keratinolytic agent also used as preservative in cosmetic products. Green chemistry, known as sustainable chemistry, is the design of products and processes eliminating the use of chemicals. It is applicable throughout a chemical product's life cycle, including its design, manufacture, use, and final disposal. The aim of this study was to synthesize SA with green chemistry methods using different amounts of wintergreen oil and to optimize the relevant steps in this path.

Materials and Methods: The SA was synthesized from natural wintergreen oil using green chemistry methods. First laboratory-scale synthesis was developed and 15 laboratory-scale synthesis trial patterns, using reaction temperature, wintergreen oil-sodium hydroxide molar ratio, sodium hydroxide-water weight ratio, reaction time and pH were performed. Purity was analysed with gas chromatography-mass spectrometry (GC-MS) and moisture analysis was performed.

Results and Conclusion: As a result of pilot production run with 1 kg, 5 kg, and three batches of 20 kg of wintergreen oil, SA was produced with a yield range of 91.06-93.92 %. The resulting SA batches had a purity of approximately 99%. This is a sufficient degree of purity for SA to be used as a raw material in cosmetics products. Filtering the SA solution using a filter press reduced crystal drying time and brought down the total production time to eight days.

Keywords: Salicylic acid, green chemistry, wintergreen oil, cosmetic

INTRODUCTION

Salicylic acid (SA) is a beta-hydroxy acid, and its name originates from the Latin word salix, which means "willow tree." As an ingredient in Aspirin, SA has numerous health benefits. It has a therapeutic effect on various skin conditions, such as acne and eczema (1). It is used in the production of cosmetic care products, such as creams, masks, shampoos and tonics (2). Moreover, SA has an exfoliating effect on skin, which helps to remove dead cells (3).

As a raw material, it is used in the production of food and textiles, as well as pharmaceuticals and cosmetic products (4). Although it has been widely used in cosmetic products in recent years due to its protective properties and dermatological effects, the SA contained in these products has been produced via industrial synthesis. In this method, phenol (which is a highly toxic chemical) is used as a raw material. The synthesis reaction of SA is presented in Figure 1. Industrial SA synthesis creates certain impurities in the end product which have toxic effects as indicated in the pharmacopeia. In contrast, the natural synthesis method uses the oil of wintergreen, which has a methyl salicylate

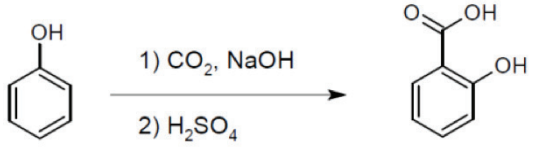

Figure 1. Industrial synthesis of SA.

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content of over 99%. Oil of wintergreen is first hydrolysed with sodium hydroxide and then further hydrolysed with hydrochloric acid to obtain crystal SA free from toxic impurities (5).

SA and its derivatives have long been recognized as important pharmacological agents. Salicin, the active ingredient in willow bark, was isolated in 1828. Hippocrates, the father of medicine, prescribed willow bark to reduce fever and pain during childbirth in the fifth century B.C. Salicylate levels have been found to be high in a variety of plant species other than willow. Another medicinal derivative known as wintergreen oil also contains methyl salicylate. Medicinal plants high in salicylates have been used by various cultures around the world for thousands of years and continue to be used today (6).

Chemical peeling is a technique used to improve, smooth, and revitalize the skin through controlled removal of epidermis/dermis, enabling healthy skin formation (7). The aim of chemical peeling is to cause damage to skin layers up to the desired depth, in order to treat various skin lesions and conditions by leveraging the increased collagen and elastin production triggered by wound healing (8). The most common chemical peeling agents are alpha-hydroxy acids (AHA) (lactic acid, glycolic acid, and fruit acids), trichloroacetic acid (TCA), beta-hydroxy acids (BHA, SA), Jessner solution, and their combinations (9). SA affects the epidermis and is generally used in the treatment of acne, acne scars, blackheads, and photo-aging, as well as in the secondary treatment of skin spots. It penetrates into the pores of the skin, preventing sebum build-up and balancing skin tone. Marczyk et al. compared the effects of 50% pyruvic acid and 30% SA peels on skin lipids and found that SA had a greater sebumetric impact than pyruvic acid (10). In the 1860s, it was discovered that SA could soften and exfoliate the stratum corneum. With its comedolytic properties, SA works to dissolve dead skin cells, and has an anti-inflammatory effect in lower concentrations, which helps to treat acne and reduce acne scars (11). SA is also a desmolytic agent because it disrupts cellular junctions rather than breaking intercellular keratin filaments (12). Imayama et al. concluded that peeling with SA can cause changes in the underlying dermal tissue without directly wounding the skin (13, 14). Its anti-inflammatory and anti-irritant properties enable SA to be well-tolerated by all skin types (15). It also soothes painful acne and sensitive skin (16).

Acne vulgaris is a common condition that can cause both physical and psychological problems, such as redness after acne, hyperpigmentation after inflammation, acne scars, and affects the quality of life. SA acts on normal keratinization, reduces inflammation, and regulates sebum production with a comedolytic action. The SA concentration used to treat acne is 0.5–5% (17). SA has been shown to reduce the lipid content of the sebocytes cell line (SEB-1) to suppress the inflammatory response in SEB-1 by inhibiting the NF-kB pathway (16). SA is also effective in the treatment of dandruff, caused by keratinocyte hyper-proliferation as it loosens the bonds between the corneocytes, allowing them to be washed away (18, 19).

Green chemistry is a novel method in chemistry that aims to minimize the environmental impact during the production and use of chemicals (20). It is based on ecological concerns and takes into account economic and technological factors. It favours the most ecologically-economically advantageous solution of existing alternatives (21).

The foundation of this philosophy was laid with the enactment of the Environmental Protection Act in the United States (US) in 1990. This act focused on the prevention of polluting waste and was followed by the establishment of the Office of Pollution Prevention and Toxics within US Environmental Protection Agency (EPA) (22). The twelve green chemistry principles were presented as the first guidebook on green chemistry by Anastas, a US EPA representative, and Warner (23). The history of green chemistry was initiated by the pollution prevention movement in 1990. Then it was formalized with the establishment of EPA in 1991"Presidential Green Chemistry" awards were given for the first time in 1996. The "Green Chemistry and Engineering" conference was first held in 1997 (24).

The goals of green chemistry are schematized in Figure 2. To achieve these goals, the principles of green chemistry include preventing waste, maintaining atom economy and synthesis of less toxic chemicals, and developing safer chemicals, safer solvents, and auxiliaries. Energy efficiency should also be maintained through the use of renewable feedstocks. Derivatization is aimed to be reduced, minimized, or avoided, as these steps require additional reagents and can cause waste. Chemical products should be designed so that when they reach the end of their useful life, they degrade into harmless degradation products and do not persist in the environment. Analytical methodologies that enable real-time, in-process monitoring and control prior to the formation of hazardous substances must be developed further. Finally substances used in a chemical process should be selected to reduce the likelihood of chemical accidents such as releases, explosions, and fires (24, 25).

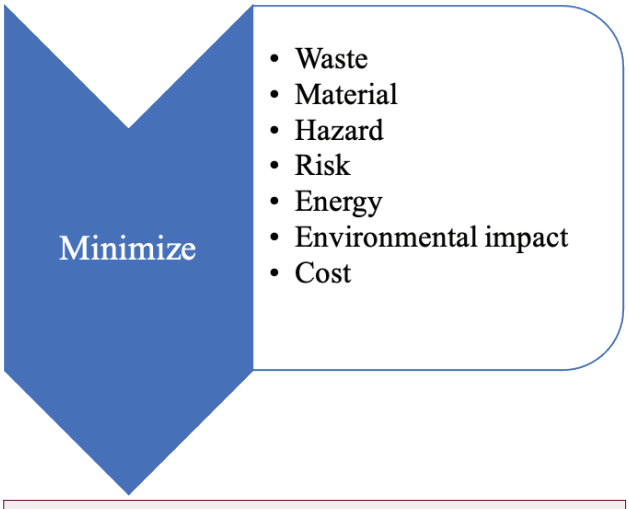

Figure 2. Goals of green chemistry.

In the light of this information, the aim of our study was to synthesize SA with green chemistry methods using different amounts of wintergreen oil and to optimize the relevant steps in this path.

MATERIALS AND METHODS

Salicylic Acid Synthesis using Green Chemistry

Developing Laboratory-Scale Synthesis

SA was synthesized from natural wintergreen oil using organic synthesis and green chemistry methods. First, sodium salicylate was synthesized and then it is crystallized as described below. The main synthesis steps were schematized in Figure 3.

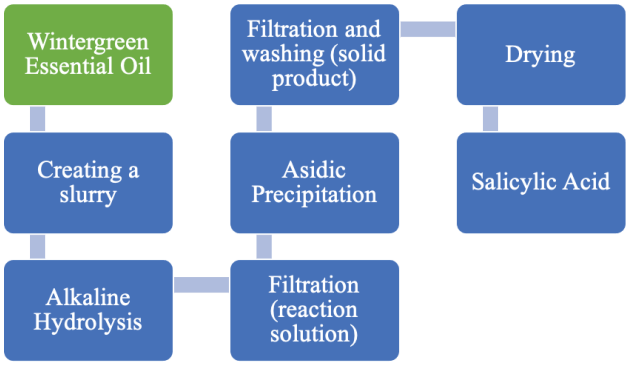

Figure 3. The main steps of SA synthesis.

Sodium Salicylate Synthesis

Sodium hydroxide solution (5M) was slowly added to wintergreen oil over 10-15 minutes. White precipitates formed. Reflux was commenced by turning on the heater and stirrer. A homogeneous solution was formed within one hour; heating continued for a further two hours for three hours of total reflux. After three hours, the solution was left to cool at room temperature. The reflux equipment system is shown in Figure 4.

Crystallization of Salicylic Acid

At room temperature, to the reaction solution the HCl solution (6M) was slowly added over 30 minutes to reduce its pH to between 2 and 1.5. The addition of HCl created an exothermic reaction. The solution was cooled continuously while the HCl was added. The solution was left overnight to allow precipitates to

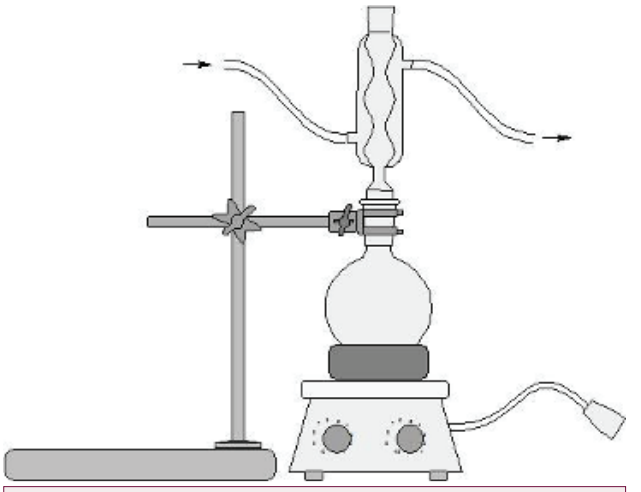

Figure 4. Reflux equipment system.

form. Then it was washed and filtered under vacuum. The crystals were washed using water and dried at 40°C for 3-4 days. The filtration equipment is shown in Figure 5 (26, 27).

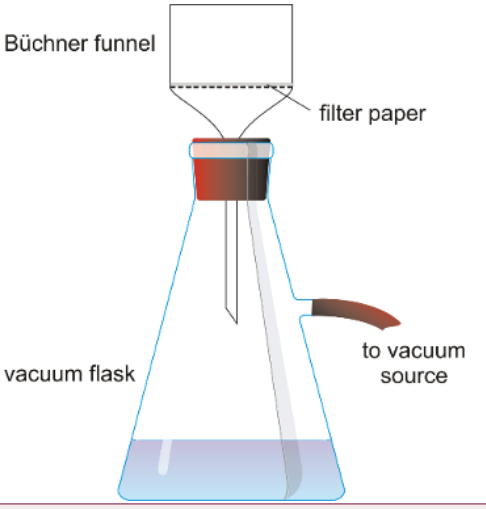

Figure 5. Filtration equipment.

Laboratory-Scale Synthesis Trial Pattern

In our studies a total of 15 trials were made, using the parameters in Table 1. The optimum parameters with the highest reac-

Table 1. Laboratory-Scale Synthesis Trial Pattern of SA.

Parameter 1	Reaction Temperature	80°C, 90°C, 100°C
Parameter 2	Wintergreen oil-sodium hydroxide molar ratio	1:3, 1:5, 1:7
Parameter 3	Sodium hydroxide-water weight ratio	1:5, 1:7, 1:10
Parameter 4	Reaction Time	2 hours, 3 hours, 4 hours
Parameter 5	pH (HCl precipitation)	1.5, 1.7, 2.0

tion yield were reaction temperature: 90°C; 1:7 wintergreen oil-sodium hydroxide molar ratio; 1:5 sodium hydroxide - water weight ratio; reaction time of 3 hours, and pH of 1.5. The resulting SA was analysed for purity using gas chromatography-mass spectrometry (GC-MS), which revealed a purity of 99%.

GC-MS Analysis

For the GC-MS analysis a HP-5ms ultra inert, 30 m x 250 µm x 0.25 µm column was used. Temperature program was arranged as follows: beginning: 60°C, final 260°C, and the temperature increase rate was 3°C per minute. Inlet temperature was 250°C and the MS detector temperature was 230°C. Analysis duration was 66.6 minutes. Helium flow rate and the split ratio were 1.1 ml/minute and 20:1, respectively.

Sample Preparation

5 mg of SA was dissolved in 1.5 ml of methanol. Injection volume was 1µl and SA retention time was 20.3 minutes. SA MS peaks were determined as 138, 120, 92, 64, 53 in molecular weight.

Moisture Analysis

The samples were placed in moisture analyser at 95°C, and it was observed that samples that were dried at 40°C for four days had a moisture content of under 0.5%.

Pilot-Scale Synthesis Optimization

Pilot-scale production was optimized by gradually increasing wintergreen oil amounts (1 kg, 5 kg, 20 kg). Synthesis parameters (caustic ratio, acid amount, drying time) were taken into account to obtain laboratory-scale synthesis data. Yield and impurity analyses were performed after each batch. The Pilot-Scale synthesis trial pattern of SA is given in Table 2.

Yield and Purity Analysis Criteria for Pilot-Scale Salicylic Acid Production

The criteria include the fulfilment of the following criteria: a yield of a minimum 80% raw material input, a minimum 95% purity as analysed by GC-MS and a maximum loss of 0.5 % on drying analysis through moisture analyser.

RESULTS

Results of Salicylic Acid Synthesis from 1 kg of Wintergreen Oil

At the end of sodium salicylate synthesis and crystallization steps, the product was dried for 14 days at 40°C to obtain 0.84 kg of SA with a yield of 92.47%. The resulting SA was analysed for purity using GC-MS, which revealed a purity of 98.25%. The product's moisture content was analysed at 95°C, showing that samples that were dried at 40°C for 14 days had a moisture content of under 0.5%.

Results of Salicylic Acid Synthesis from 5 kg of Wintergreen Oil

4.18 kg of SA with a yield of 91.96% was obtained. The resulting SA was analysed for purity using GC-MS, which revealed a purity of 97.95%. Moisture analysis revealed that samples dried at 45°C for 10 days had a moisture content of under 0.5%.

Results of Salicylic Acid Synthesis from 20 kg of Wintergreen Oil (20 kg batch)

For the first batch, 17.07 kg of SA with a yield of 93.92% was obtained. The resulting SA was analysed for purity using GC-MS, which revealed a purity of 99.16%. The product's moisture content was analysed at 95°C, showing that samples that were dried at 50°C for 14 days had a moisture content of under 0.5%.

For the second batch, 16.93 kg of SA with a yield of 93.19% was obtained. The resulting SA was analysed for purity using GC-MS, which revealed a purity of 99%. Moisture content was analysed at 95°C, showing that samples that were dried at 50°C for 7 days had a moisture content of under 0.5%.

For the third batch, 16.96 kg of SA with a yield of 93.34% was obtained. The resulting SA was analysed for purity using GC-MS, which revealed a purity of 99%. Moisture content was analysed at 95°C, showing that samples that were dried at 45°C for 7 days had a moisture content of under 0.5%. The pilot production results are given in Table 3.

Table 2. Pilot-Scale Synthesis Trial Pattern of SA.

Parameter 1	Amount of wintergreen oil	1kg, 5 kg, 20 kg
Parameter 2	SA drying temperature	40°C, 45°C, 50°C
Parameter 3	SA drying time	7 days, 10 days, 14 days

Table 3. Pilot Production Results of SA.

1 kg batch	Total production time 15 days	Yield 92.47%, purity 98.25%
5 kg batch	Total production time 15 days	Yield 91.96%, purity 97.95%
First 20 kg batch	Total production time 15 days	Yield 93.92%, purity 99.16%
Second 20 kg batch (press filtered)	Total production time 8 days	Yield 93.19%, purity 99%
Third 20 kg batch (press filtered)	Total production time 8 days	Yield 93.34%, purity 99%

Pilot production runs with 1 kg, 5 kg and three batches of 20 kg of wintergreen oil produced SA with a yield range of 91.06-93.92% using green chemistry methods. The resulting SA batches had a purity of approximately 99%. This is a sufficient degree of purity for the SA to be used as a raw material in cosmetics products. Filtering the SA solution via a filter press had a reduced crystal drying time and brought down total production time to eight days.

DISCUSSION

SA is one of the most common active ingredients used in cosmetic products. It is an organic compound. It is a colourless crystal found naturally in various plants, such as willow bark or wintergreen. SA used in skin care products can be either natural or synthetic.

According to a report by the Regional Network Coordinating Organizations (RNCOs), which is an Indian based market research company, the world cosmetics market was valued at $233 billion in 2012, and is projected to reach $ 480,4 billion by 2030, with a compound annual growth rate of 4.6% (28, 29). In response to such anticipated growth, cosmetic brands are expected to keep abreast of customer needs and develop innovative products if they want to maintain their position in the market (28).

In 2012, global SA consumption was at 79,725 tonnes, and this figure is expected to climb to 149,652 tonnes in 2023. This indicates a compound annual growth rate of 6.5%. Total global sale revenue of SA was $239.5 million in 2012, and this is expected to rise to $546.8 million in 2023. This indicates a compound annual growth rate of 8.6%. The regional breakdown of the SA market for the year 2013 was as follows: North America 27.9%, Europe 34.9%, Asia-Pacific 25.3%, and other regions 11.9%. Natural cosmetic products account for around 1% of global cosmetics market (28, 30).

In addition to skin care products, SA is also included in hair care formulations to treat excessive oil and dandruff (31). It cleanses the scalp. It is used as an anti-dandruff agent in hair products (conditioners, shampoos) and in baby shampoos to prevent cradle cap. It is also used as a preservative to extend the shelf life of products (32). It inhibits the growth of various types of bacteria. SA is considered safe when used as a preservative in cosmetic products at a concentration of 0.5%, according to the Scientific Committee on Consumer Safety. SA has a strong antifungal effect. SA produced protein leakage into the medium, significant lipid degradation, and intracellular disarray in the pathogen. Having keratolytic effect and dissolving the superficial layers of the epidermis, SA has an important therapeutic effect on oily and problematic skin. It is also used in medicine for its analgesic and anti-inflammatory properties (33).

Using alternative solvents, reducing waste, increasing the efficiency of the different processes, improving economy in energy, and using safe chemicals are the main concepts of green chemistry. Solvents are required in these reactions to dissolve solids, enable transfer of material (extraction), stabilize transition states and to facilitate precipitation. Non-toxicity alone does not indicate that a certain product is compatible with green chemistry. Solvent reclamation, solubility, lack of toxic formations, atom efficiency, separation of product and solvent and ineffectiveness of solvent on end product are required factors to be compatible with green chemistry. Water as a molecular solvent offers high solubility with polar compounds besides being clean, cheap and having low reactivity. On the other hand, organic solvents are toxic, costly and flammable. 15 million kg of organic solvent is used globally every year. The primary mission of green chemistry is to find alternatives to these solvents.

Different from our study, Molleti and Yadav (34) prepared a new sulphated Fe_2O_3–ZrO_2 catalyst with altered iron loadings using the combustion technique and utilized in methyl salicylate preparation from SA and dimethyl carbonate. The methyl salicylate produced was reported to be widely useable in food and pharma industries. They also evaluated the effect of different kinetic parameters on the esterification rate of SA. They reported that optimum conditions for the 99% conversion of SA with the 100% selectivity to be 120°C after 150 min at a molar ratio of 1:10, SA to dimethyl carbonate.

In our study, filtering the SA solution via a filter press led to a reduced crystal drying time, and brought down total production time to eight days. As the SA production size increases (from 1 kg to 20 kg), one of the biggest problems is getting more moist solids after filtration. The filter press device is a special filtering device. During filtration, air is applied to the crystals, resulting in drier solids. In this way, the drying time of the solid is significantly reduced. Accordingly one of the most important parameters of green chemistry is to shorten the process time.

To obtain a marketable product, it is essential to create the necessary conditions for pilot production. Data obtained from laboratory-scale synthesis is used to increase production gradually to factory-scale. For this purpose, in our study, different batches of (1 kg, 5 kg, 20 kg) oil of wintergreen was prepared for the production of SA. Our results revealed a sufficient degree of purity for the SA to be used as a raw material in cosmetics products. Accordingly evaluation of production purity with GC-MS stands out as an important feature to support the results of our study.

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

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REFERENCES

1. Calvisi, Lucia. Efficacy of a combined chemical peel and topical salicylic acid-based gel combination in the treatment of active acne. J Cosmet Dermatol 2021; 20: 2-6. [CrossRef]
2. Ekinci D, Sentürk M, Küfrevioğlu Öİ. Salicylic acid derivatives: synthesis, features and usage as therapeutic tools. Expert Opin Ther Pat 2011; 21(12): 1831-41. [CrossRef]
3. Arif T. Salicylic acid as a peeling agent: a comprehensive review. Clin Cosmet Investig Dermatol 2015; 8: 455-61. [CrossRef]
4. Krishika Sambyal, Rahul Vikram Singh. Production of salicylic acid; a potent pharmaceutically active agent and its future prospects. Crit Rev in Biotech 2021; 41(3): 394-405. [CrossRef]
5. Parker D, Martinez C, Stanley C, Simmons J, McIntyre IM. The analysis of methyl salicylate and salicylic acid from Chinese herbal medicine ingestion. J Anal Toxicol 2004; 28(3): 214-6. [CrossRef]
6. Yeasmin F, Choi HW. Natural salicylates and their roles in human health. Int J Mol Sci 2020; 21(23): 9049. [CrossRef]
7. Rendon MI, Berson DS, Cohen JL, Roberts WE, Starker I, Wang B. Evidence and considerations in the application of chemical peels in skin disorders and aesthetic resurfacing. J Clin Aesthet Dermatol 2010; 3(7): 32-43.
8. Soleymani T, Lanoue J, Rahman Z. A Practical Approach to Chemical Peels: A Review of Fundamentals and Step-by-step Algorithmic Protocol for Treatment. J Clin Aesthet Dermatol. 2018; 11(8): 21-28.
9. O'Connor AA, Lowe PM, Shumack S, Lim AC. Chemical peels: A review of current practice. Australas J Dermatol 2018; 59(3): 171-81. [CrossRef]
10. Marczyk B, Mucha P, Budzisz E, Rotsztejn H. Comparative study of the effect of 50% pyruvic and 30% salicylic peels on the skin lipid film in patients with acne vulgaris. J Cosmet Dermatol 2014; 13: 15-21. [CrossRef]
11. Lévêque JL, Corcuff P, Gonnord G, Montastier C, Renault B, Bazin R, Piérard G, Poelman MC. Mechanism of action of a lipophilic derivative of salicylic acid on normal skin. Skin Res Technol 1995; 1(3): 115-22. [CrossRef]
12. Zhao Q, Dai C, Fan S, Lv J, Nie L. Synergistic efficacy of salicylic acid with a penetration enhancer on human skin monitored by OCT and diffuse reflectance spectroscopy. Sci Rep 2016; (6) 34954. [CrossRef]
13. Imayama S, Ueda S, Isoda M. Histologic changes in the skin of hairless mice following peeling with salicylic acid. Arch Dermatol. 2000; 136: 1390-5. [CrossRef]
14. Shin SB, Hu H, Xi K. US9474702B2 Cosmetic use of salicylic acid derivatives, 2016.
15. Randjelovic P, Veljković S, Stojiljković N, Sokolovic D, Ilić I, Laketić D et al. The beneficial biological properties of salicylic acid. Acta Facultatis Medicae Naissensis 2015; (32): 259-65. [CrossRef]
16. Lu J, Cong T, Wen X, Li X, Du D, He G, Jiang X. Salicylic acid treats acne vulgaris by suppressing AMPK/SREBP1 pathway in sebocytes. Exp Dermatol 2019; 28(7): 786-94. [CrossRef]
17. Kantikosum K, Chongpison Y, Chottawornsak N, Asawanonda P. The efficacy of glycolic acid, salicylic acid, gluconolactone, and licochalcone A combined with 0.1% adapalene vs adapalene monotherapy in mild-to-moderate acne vulgaris: a double-blinded within-person comparative study. Clin Cosmet Investig Dermatol 2019; 12: 151-61. [CrossRef]
18. Sanfilippo, A. & English III, Joseph. An overview of medicated shampoos used in dandruff treatment. P and T 2006; 31: 396-400.
19. Sushant S, Minakshi S, Yashwant S, Mamta S, Ashish P. Dandruff and Chemo-Antidandruff Agents. Res J Topical and Cosmetic Sci 2011; 2 (1): 1-10.
20. Anastas, PT, Warner JC. Green Chemistry: Theory and Practice, Oxford University Press, 1998.
21. Alfonsi K, Colberg J, Peter JD, Fevig T, Jennings S, Johnson TA, et. al. Green chemistry tools to influence a medicinal chemistry and research chemistry based organization. Green Chem 2008; 10, 31-6. [CrossRef]
22. Linthorst, Arjan. An Overview: Origins and development of green chemistry. Found Chem 2010; (12): 55-68. [CrossRef]
23. Anastas PT, Warner JC. Green Chemistry: Theory and Practice. Oxford University Press, New York, 1998.
24. Marco B, Rechelo B, Tótoli E, Kogawa A, Salgado H. Evolution Of Green Chemistry And Its Multidimensional Impacts: A Review. Saudi Pharm J 2018; (27): 1-8. [CrossRef]
25. Sheldon RA. Fundamentals of green chemistry: Efficiency in reaction design. Chem Soc Rev 2012; 41(4): 1437-51. [CrossRef]
26. Bruce DW, Perona JJ. Kinetics of the Hydrolysis of Methyl Salicylate by Sodium Hydroxide. Ind Eng Chem Process Des Dev 1985; 24: 62-5. [CrossRef]
27. Crockatt M, Urbanus JH, Konst PM, De Koning MC. Method to prepare phenolics from biomass. 2016, WO2016/114668A1.
28. Salicylic acid market analysis by application (Pharmaceuticals, Food & Preservative, Cosmetics) and segment forecasts to 2024-Grand view research (Available from: https://www.grandviewresearch.com/industry-analysis/salicylic-acid-market-analysis).
29. Precedence Research, Recently Announced Report on "Cosmetics Market Size, Share, Growth, Trends, Regional Outlook, Industry Analysis and Forecast 2021 - 2030" (Available from: https://www.globenewswire.com/news-release/2021/10/05/2308851/0/en/Cosmetics-Market-Size-to-Worth-Around-US-480-4-Bn-by-2030.html).
30. Lekakh O, Mahoney AM, Novice K, Kamalpour J, Sadeghian A, Mondo D, et al. Treatment of acne vulgaris with salicylic acid chemical peel and pulsed dye laser: A split face, rater-blinded, randomized controlled trial. J Lasers Med Sci 2015; 6(4): 167-70. [CrossRef]
31. Schwartz J, Deangelis YM, Dawson T. Dandruff and Seborrheic Dermatitis: A Head Scratcher. 2012.
32. Ikarashi Y, Uchino T, Nishimura T. Analysis of preservatives used in cosmetic products: salicylic acid, sodium benzoate, sodium dehydroacetate, potassium sorbate, phenoxyethanol, and parabens. Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku 2010; 128: 85-90.
33. SCCS 1601/18. Opinion on salicylic acid (CAS 69-72-7)- Submission I - Corrigendum of 20-21 June 2019 (Available from: https://ec.europa.eu/health/document/download/b9f205cb-07d0-4eca-895a-87de952d5b6d_en).
34. Molleti J, Yadav GD. Green synthesis of methyl salicylate using novel sulfated iron oxide-zirconia catalyst. Clean Techn Environ Policy 2019; 21: 533-45. [CrossRef]

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ORIGINAL ARTICLE

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Dye Ligand Affinity Nanoparticles for the Depletion of Biomolecules in Proteomics

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ABSTRACT

Objective: Serum proteins are indicators for certain diseases. However, detection of the biomarkers is difficult because the more abundant proteins mask the less abundant ones. The depletion of abundant serum proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers. Dye ligands have attracted great attention because of their cost-effectiveness, easy immobilization, stability, and high binding capacity. Due to these advantageous properties, dye ligands have also been chosen as an alternative for biological ligands such as antibodies, enzymes, etc.

Materials and Methods: Poly (ethylene glycol dimethacrylate) [p (EGDM)] nanoparticles were prepared using the surfactant-free emulsion polymerization technique. Then, Reactive Red 120 (RR 120) dye was immobilized to nanoparticles in a nucleophilic substitution reaction. The RR120 attached nanoparticles were characterized.

Results: The size/size distribution of p (EGDM) nanoparticles was measured with a Zeta sizer. The scanning electron microscope (SEM) images were found to support a measurement of around 100 nm. The maximum adsorbed amount of albumin was observed at pH 6.0. The maximum depleted albumin concentration was found to be 453.9 mg/g nanoparticles according to the experimental results. Desorption studies were carried out by addition of 0.5 M of KSCN to the albumin solutions. The desorption results showed that the binding of albumin to the nanoparticle was reversible.

Conclusion: Our results demonstrated that dye attached nanoparticles have the potential for depleting albumin from serum in proteomics.

Keywords: Proteomics, dye ligand, nanoparticles, Reactive Red 120, biomolecules, albumin

INTRODUCTION

Proteomics is focused on the study of proteins, particularly their structures and functions. Serum proteins are indicators for certain diseases and these proteins have been used as biomarkers for these disorders (1).

Serum contains 60–80 mg of protein/mL, in addition to various molecules, including lipids, electrolytes, etc. (2). The total number of proteins is approximately ten thousand, but this number can reach several times this amount in normal and diseased cells as each protein undergoes many post-translational modifications. However, the more abundant proteins make proteome analysis very challenging because these proteins mask the less abundant ones (1, 3, 4). Indeed, one major challenge of plasma protein analysis is that as few as 0.2 % of the proteins make up 99 % of the total protein content of serum. Albumin, for example, represents more than half of the whole blood protein mass in serum (5). Nevertheless, the timely detection of misexpressed proteins in the early stages of a given disease is extremely challenging (6). This is especially important in proteomic studies where potential biomarkers are investigated. Therefore, the first step when examining serum proteome analysis in general is to reduce sample complexity (3).

It is very important that any methods developed in the depletion of highly abundant proteins should be low-cost and involve high-throughput techniques. There have been

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many techniques developed for the depletion of albumin in the literature. Among these techniques, affinity technologies draw much attention.

Dye ligands have attracted great attention because of their cheapness, easy immobilization properties, stability, and high adsorption capacity. Due to these advantageous properties, dye ligands have also been chosen as an alternative for the biological ligands such as antibodies, enzymes, etc. (6-11). These dye ligands are structurally very similar to some biological molecules (cofactor, substrate, etc). Because of this similarity, dye ligands can easily interact with the active center of some enzyme structures. Therefore, these dye ligands are called affinity ligands. Many reactive dyes which are also used as textile dyes have been used for the adsorption of proteins. These dyes generally contain a reactive group (mono- or dichloro triazine ring) with a chromophore group (azo, anthroquinone or phthalocyanine). Due to the complex structures of dye molecules and protein molecules, the interactions between them are usually secondary interactions. In general, hydrophobic interactions, electrostatic bonds and hydrogen bonds are quite dominant among these interactions (12).

Adsorption capacity of polymeric support material increases when the particle size of the support is decreased (13-15). In the case of the surface adsorption concept, when the particle size decreases, its surface area tends to be wider and therefore shows higher adsorption capacities (16). Synthesis and development of new nano-sized materials is a way to increase the adsorption capacity. Also, the physical and chemical properties of the nano-sized polymer demonstrate atypical change because of their small size and large surface area (17-19).

Various sorbent systems containing different dye ligands were used for the depletion, purification, and separation of albumin. Reactive Green HE-4BD containing hollow fibers (20); Cibacron Blue F3GA immobilized poly (GMA) microbeads and chitosan microspheres (21,22); and Reactive Green 19 immobilized p (HEMA) cryogel disks could be listed as examples (23).

In this study, firstly, RR 120 dye was attached to poly(ethylene glycol dimethacrylate) [p (EGDM)] nanoparticles. At the end of the synthesis procedure, characterization studies were completed. Then, reusability of the RR 120 attached p (EGDM) nanoparticles was also investigated.

MATERIALS AND METHODS

RR 120 Attachment to the p (EGDM) Nanoparticles

P (EGDM) nanoparticles were synthesized using a surfactant-free emulsion polymerization technique (24). Dye ligand RR 120 was covalently attached to the synthesized nanoparticles. For this, 70.0 mL of RR 120 solution was added on 0.1 g p (EGDM) nanoparticles. (3.0 mg/ml; in 4.0 g of NaOH) for 4 h. After this incubation period, dye attached nanoparticles were washed with distilled water and methanol several times in order to remove physically attached RR 120 molecules. RR 120-attached poly (EGDM) nanoparticles were stored at 4°C.

Characterization of RR 120 Attached Nanoparticles

Fouirer transform infrared spectroscopy (FTIR) analysis was performed to show the binding of RR 120 onto p (EGDM) nanoparticles. For this purpose, 0.1 g nanoparticles were mixed with 0.1 g of KBr homogeneously, and then pressed into pellets. The FTIR spectra of dye ligand-bound and non-dye ligand-bound nanoparticles were examined using an FTIR (Perkin Elmer Spectrum 100, USA).

Assessment of surface morphology and size of the synthesized nanoparticles was carried out using a scanning electron microscope (SEM). The SEM images were obtained by coating the dry p (EGDM) nanoparticles with a thin gold layer (Philips, XL-30S FEG, The Netherlands). Particle size of the synthesized nanoparticles was analyzed with a nano zetasizer (Nanos, Malvern Instruments, London, UK).

Adsorption of Bovine Serum Albumin (BSA) onto RR 120 Attached Nanoparticles

In our study, BSA binding studies were carried out in a batch system. In this system, synthesized nanoparticles were mixed with BSA solution, and the adsorption experiment was conducted at 25°C for 2 h with a 100 rpm stirring rate. The effects of time, medium pH, BSA concentration, and temperature on the BSA binding capacity of RR 120 attached nanoparticles were also investigated. For these purposes, some adsorption conditions were changed. For example, binding experiments were performed for different adsorption times (5-150 minutes) to show the effect of time for BSA adsorption. In addition, the pH of the solution was changed between 4.0 and 8.0 using various buffer systems. The effect of BSA concentration on adsorption was investigated by changing the BSA concentration in the range of 0.2-8.0 mg/mL and performing binding experiments. Adsorbed BSA amount was calculated by the determination of the initial and final BSA concentration using the Bradford method (25).

Desorption Studies

To evaluate the reusability of the RR 120 attached p (EGDM) nanoparticle, first the adsorbed BSA was removed. To do this, a 0.5 M KSCN solution was used as a desorption agent. The nanoparticles loaded with BSA were incubated with the 0.5 M of KSCN solution for 1 h at 25°C with constant shaking at 150 rpm. BSA desorption ratios from the dye attached nanoparticles were calculated by the following equation:

$$\text{Desorption ratio } (\%) = \frac{\text{Desorbed BSA amount}}{\text{Adsorbed BSA amount}} \text{ x } 100$$

When the reusability of the RR 120 bound nanoparticles were evaluated, experiments were performed over five adsorption/desorption cycles with the same nanoparticles.

RESULTS AND DISCUSSION

Recently, nanotechnology has become an important research area and very significant developments have occurred in various areas of science and technology. When considering the

biotechnology fields, collaboration between nanomaterials and biomolecules has attracted great interest (26). Their wide surface areas present great binding spaces for biomolecules and they can also be easily derivatized with a number of ligands. Even with their small particle size, nonporous nanoparticles can bind over 100 mg of biomolecules per gram of wet particles (27).

Especially in recent years, when the literature is examined, it was seen that nano-sized polymeric materials have frequently been used for the isolation and purification of biomolecules. This is due to their extremely high surface area, nano-sized materials that offer large surface area for increased adsorption capacity of biomolecules. Functional surface groups of the nano-sized polymers allow easy derivatization with various ligands which have different character and functionalities. Dye ligands have been used as an alternative for biological ligands such as antibodies, enzymes due to cost-effective, easy immobilization, stability and high binding capacity.

After the nanoparticles were synthesized by emulsion polymerization technique, RR 120 was covalently bonded to the surfaces of nanoparticles in a nucleophilic substitution reaction between the triazine chloride of RR 120 and hydroxyl groups of EGDM. The hypothetical representation of p (EGDM) nanoparticles with RR 120 attached is shown in Figure 1.

The dye ligand adsorption capacity of p (EGDM) nanoparticles increases with increasing the dye concentration. Nanoparticles

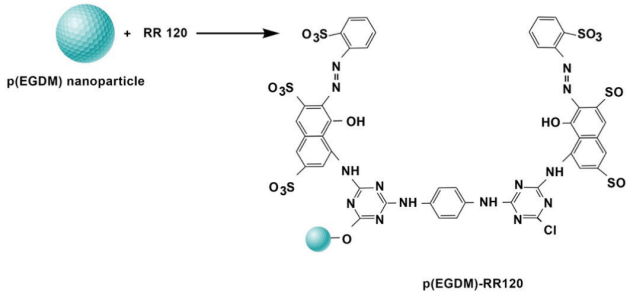

Figure 1. Schematic presentation of RR 120 attached p (EGDM) nanoparticles.

reach the maximum adsorption capacity whereas free –OH groups on the surface of nanoparticles reach saturation. Figure 2 demonstrates the FTIR spectra of RR 120, RR 120 attached nanoparticles and p (EGDM).

The O-H stretching vibration band was observed to be 3200–3600 cm^{-1}. With the addition of RR 120 to p (EGDM), absorption was significantly increased because of the existence of N-H bending in the RR 120 structure. At p (EGDM) polymer spectrum, the stretching vibrations band of carbonyl (C=O) groups was observed around 1720. Characteristic stretching vibrations bands of ester (C-O) were around 1000-1200 cm^{-1}. The bands were seen at 1080 cm^{-1} and 1160 cm^{-1} in the p (EGDM)-RR 120 and RR 120 spectrums were due to the symmetrical stretching of the S=O bond and the asymmetric stretching of the S=O bond in RR 120. As a result, the obtained spectra supports that RR 120 participates in the p (EGDM) nanoparticles.

The size and shape of RR 120 attached nanoparticles were determined by examining the SEM photographs (Figure 3). When

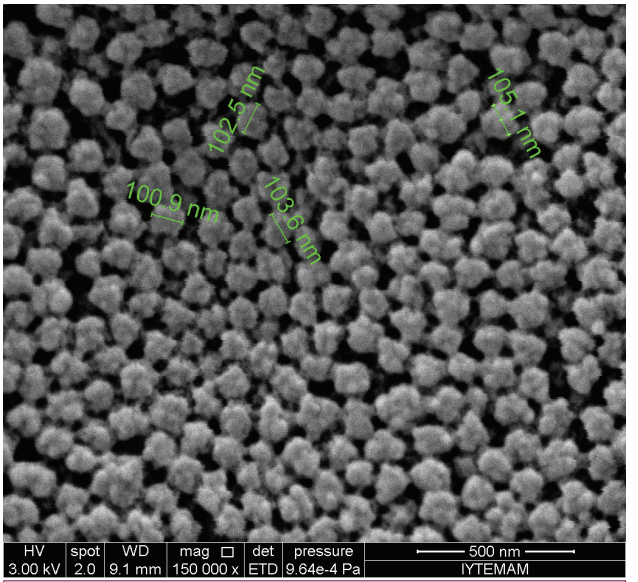

Figure 3. SEM images of p (EGDM)-RR 120 nanoparticles.

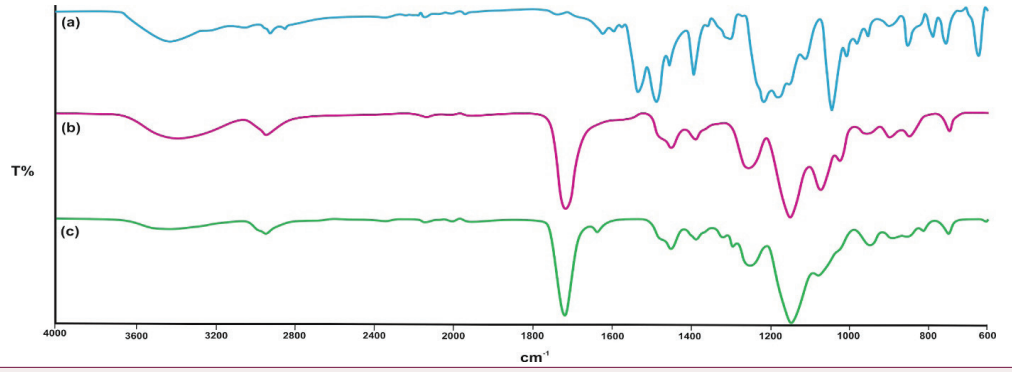

Figure 2. (a) RR120, (b) p (EGDM)-RR120, (c)p (EGDM) spectrum of FTIR.

the SEM photographs were investigated, it was seen that the nanoparticles were relatively spherical, non-porous and approximately 100 nm in width.

In addition, the size/size distribution of p (EGDM) nanoparticles was measured with a Zeta sizer. A measurement of approximately 100 nm was found to support the size recorded in the SEM images (Figure 4).

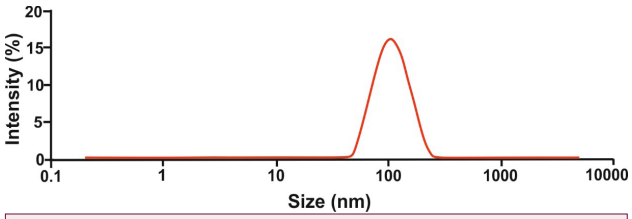

Figure 4. Zeta-size analysis of the RR 120 attached p (EGDM) nanoparticles.

To determine the effect of time on albumin binding, adsorption studies on dye-attached nanoparticles were performed between 0 and 150 minutes. When Figure 5 is examined, it is seen that albumin adsorption increases over time and reaches a plateau value at 120 minutes. Since it reached the plateau value in 120 minutes, all experiments were carried out for 120 minutes.

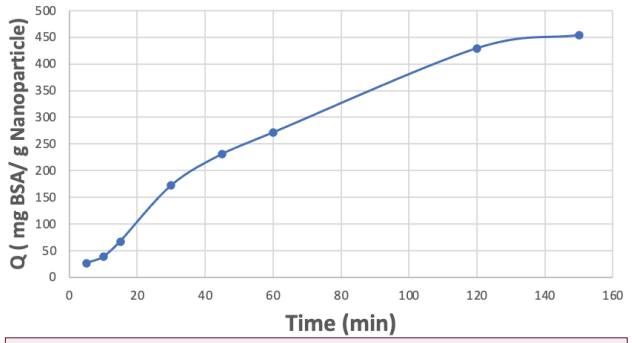

Figure 5. BSA adsorption and time curve. (BSA initial: 2mg/mL, pH:6, T=25°C).

As shown in figure 5, BSA adsorption and time studies were done at 5, 10, 15, 30, 45, 60, 120 and 150 minutes (BSA initial: 2 mg/mL, pH:6, T=25°C). Adsorption capacity values Q increased with time and reached a maximum value (453.9 mg/g) in these conditions.

Albumin adsorption experiments were performed with various pH and the pH effect on albumin binding is shown in Figure 6.

Figure 6 shows the pH effect on BSA adsorption to dye attached p (EGDM) nanoparticles. According to the graph, from the acidic to the neutral region, the adsorption capacity of p (EGDM-RR120) nanoparticle increases. Maximum BSA adsorption was found at pH: 6 to be 453.9 mg/g. The important point here is the non-specific adsorption to the nanoparticles. According to Figure 6, the non-specific adsorption to the p (EGDM) nanoparticles was negligible. The interactions between albumin and RR 120 molecules attached on the nanoparticle surface are weak interactions. When the structure of albumin and RR 120 molecules is examined, hydrophobic, electrostatic and/or hydrogen bonds may occur between these two molecules. These interactions may increase further due to the presence of sulfonate, amine, and cyclic hydrophobic groups on the dye molecules and a few ionizable groups on the amino acid side chains on the albumin molecule.

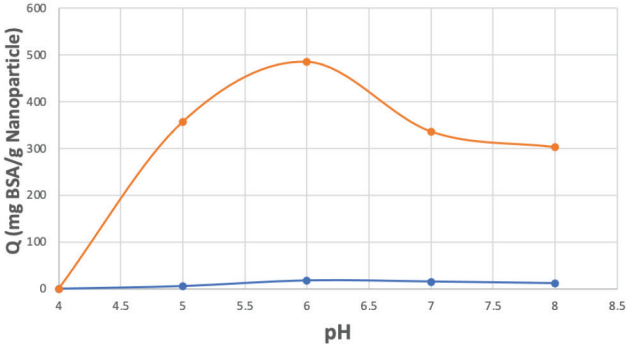

Figure 6. pH effect on BSA binding (Concentration of BSA: 2mg/mL, adsorption time: 2 hours) (Blue line: non-specific adsorption; red line: RR 120 attached p (EGDM)).

The effect of albumin concentration on the binding capacity of RR 120 attached p (EGDM) nanoparticles is shown in Figure 7. When the initial albumin concentration was increased, the adsorbed amount of albumin onto dye-attached nanoparticles increased rapidly. The saturation value was 6.0 mg/mL of albumin concentration and generally takes place in affinity adsorption studies because all active biomolecules binding regions are busy with previously bound biomolecules. It can also be concluded from Figure 7 that affinity between the RR 120 and albumin molecules was very high.

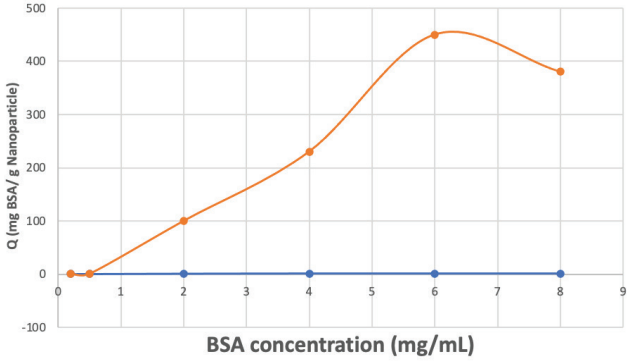

Figure 7. Effect of concentration on BSA binding (pH: 6, adsorption time: 2 hours) (Blue line: non- specific adsorption; red line: RR 120 attached p (EGDM)).

Figure 8 presents temperature effect on the albumin binding onto RR 120 attached p (EGDM) nanoparticles. As can be seen

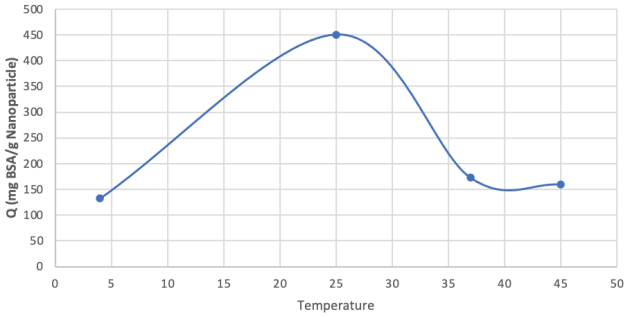

Figure 8. Temperature effect on the BSA adsorption (Concentration of BSA: 1mg/mL, pH: 6, adsorption time: 2 hours).

in Figure 8, the amount of adsorbed albumin increased with increasing temperature. This adsorption behavior can be explained by the hydrophobic interaction between the albumin and RR 120 attached nanoparticles. In hydrophobic interaction chromatographic techniques, the adsorbed amount of adsorbent generally increases with increasing temperature. Albumin adsorption capacity of the RR 120 attached nanoparticles decreased at temperatures higher than 25°C. When considering the protein adsorption, the adsorbed amount of proteins generally decreases because of the three dimensional conformational changes occurred at high temperatures.

The results of the presented study show that RR 120 attached nanoparticles are very valuable polymeric materials which can be used for adsorption of BSA and therefore can be applied in the depletion of albumin. The high albumin adsorption capacity of the prepared nanoparticles can increase its usage for biotechnological and biomedical applications.

As we mentioned before, in the experiments in which the reusability of RR 120 bound nanoparticles were evaluated, the experiments were performed by performing five adsorption/desorption cycles with the same nanoparticles. The albumin adsorbed RR 120 attached nanoparticles were desorbed with 0.5 M KSCN. Desorption rates were found to be between 70-85 %. It should be noted that at the end of the applied five adsorption/desorption cycles, there was no significant decrease in the adsorption capacity of albumin to dye-attached nanoparticles (Figure 9).

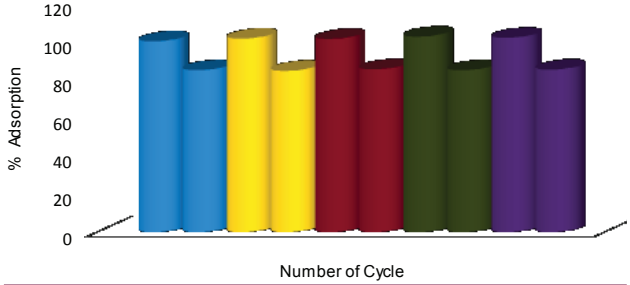

Figure 9. Reusability of p (EGDM)-RR 120 nanoparticle (Desorption agent: 0.5 M KSCN, concentration of BSA: 1mg/mL, pH: 6, adsorption time: 2 hours, desorption time: 1 hours).

CONCLUSIONS

In our study, RR 120 was used for the affinity adsorption of albumin which is a highly abundant protein in serum. p (EGDM) nanoparticles were synthesized as a depletion material and derivatized with RR 120. The effect of pH, albumin concentration, and temperature on albumin adsorption onto RR 120 attached nanoparticles was investigated. Our findings from this study indicate that RR 120 attached nanoparticles can be easily used for the adsorption and depletion of albumin from plasma/serum. This nanoparticle, which was developed for the first time in our study for albumin depletion, has the advantages of high binding capacity and relatively low cost compared to other systems.

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

Peer-review: Externally peer-reviewed.

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REFERENCES

1. Andaç M, Denizli A. Affinity-recognition-based polymeric cryogels for protein depletion studies. Rsc Advances 2014; 4(59): 31130-41. [CrossRef]
2. Bellei E, Bergamini S, Monari E, Fantoni LI, Cuoghi A, Ozben T, et al. High-abundance proteins depletion for serum proteomic analysis: concomitant removal of non-targeted proteins. Amino Acids 2011; 40(1): 145-56. [CrossRef]
3. Göktürk I, Tamahkar E, Yılmaz F, Denizli A. Protein depletion with bacterial cellulose nanofibers. J Chromatogr B 2018; 1099: 1-9. [CrossRef]
4. Borberg E, Pashko S, Koren V, Burstein L, Patolsky F. Depletion of Highly Abundant Protein Species from Biosamples by the Use of a Branched Silicon Nanopillar On-Chip Platform. Anal Chem 2021; 93(43): 14527-36. [CrossRef]
5. Siegmund R, Kiehntopf M, Deufel T. (2009). Evaluation of two different albumin depletion strategies for improved analysis of human CSF by SELDI-TOF-MS. Clin Biochem 2009; 42(10-11): 1136-43. [CrossRef]
6. da Costa JP, Santos PS, Vitorino R, Rocha-Santos T, Duarte AC. How low can you go? A current perspective on low-abundance proteomics. TrAC Trends Anal Chem 2017; 93: 171-82. [CrossRef]
7. Uygun DA, Akduman B, Uygun M, Akgöl S, Denizli A. Purification of papain using reactive green 5 attached supermacroporous monolithic cryogel. Appl Biochem Biotechnol 2012; 167(3): 552-63. [CrossRef]
8. Akduman B, Uygun M, Çoban EP, Uygun DA, Bıyık H, Akgöl S. Reversible immobilization of urease by using bacterial cellulose nanofibers. Appl Biochem Biotechnol 2013; 171(8): 2285-94. [CrossRef]
9. Uygun M, Uygun DA, Altunbaş C, Akgöl S, Denizli A. Dye attached nanoparticles for lysozyme adsorption. Sep Sci Technol 2014; 49(8): 1270-8. [CrossRef]

10. Poddar S, Sharmeen S, Hage DS. Affinity monolith chromatography: a review of general principles and recent developments. Electrophoresis 2021; 42(24): 2577-98. [CrossRef]
11. Andac M, Galaev I, Denizli A. Dye attached poly (hydroxyethyl methacrylate) cryogel for albumin depletion from human serum. J Sep Sci 2012; 35(9): 1173-82. [CrossRef]
12. Wongchuphan R, Tey BT, Tan WS, Taip FS, Kamal SMM, Ling TC. Application of dye-ligands affinity adsorbent in capturing of rabbit immunoglobulin G. Biochem Eng J 2009; 45(3): 232-8. [CrossRef]
13. Öztürk N, Tabak A, Akgöl S, Denizli A. Newly synthesized bentonite-histidine (Bent-His) micro-composite affinity sorbents for IgG adsorption. Colloids Surf A. Physicochem and Eng Asp 2007; 301(1-3): 490-7. [CrossRef]
14. Alpay P, Uygun DA. Usage of immobilized papain for enzymatic hydrolysis of proteins. J Mol Catal B: Enzymatic 2015; 111: 56-63. [CrossRef]
15. Akgöl S, Öztürk N, Denizli A. New generation polymeric nanospheres for lysozyme adsorption. J Aappl PolymSci 2010;115(3): 1608-15. [CrossRef]
16. Türkmen D, Öztürk N, Akgöl S, Elkak A, Denizli A. Phenylalanine containing hydrophobic nanospheres for antibody purification. Biotechnol Prog 2008; 24(6): 1297-1303. [CrossRef]
17. Liao MH, Chen DH. Fast and efficient adsorption/desorption of protein by a novel magnetic nano-adsorbent. Biotechnol Lett 2002; 24(22): 1913-7. [CrossRef]
18. Hegedűs I, Nagy E. Improvement of chymotrypsin enzyme stability as single enzyme nanoparticles. Chem Eng Sci 2009; 64(5): 1053-60. [CrossRef]
19. Uygun DA, Çorman ME, Öztürk N, Akgöl S, Denizli A. Poly (hydroxyethyl methacrylate-co-methacryloylamidotryptophane) nanospheres and their utilization as affinity adsorbents for porcine pancreas lipase adsorption. Mater Sci and Eng: C 2010; 30(8): 1285-90. [CrossRef]
20. Yavuz H, Denizli A. Dye affinity hollow fibers for albumin purification. Macromol Biosci 2004; 4(2): 84-91. [CrossRef]
21. Altıntaş EB, Denizli A. Efficient removal of albumin from human serum by monosize dye-affinity beads. J Chromatog B 2006; 832(2): 216-23. [CrossRef]
22. Zhang J, Zhang Z, Song Y, Cai H. Bovine serum albumin (BSA) adsorption with Cibacron Blue F3GA attached chitosan microspheres. React Funct Polym 2006; 66(9): 916-23. [CrossRef]
23. Tuzmen N, Kalburcu T, Uygun DA, Akgol S, Denizli A. A novel affinity disks for bovine serum albumin purification. Appl Biochem Biotechnol 2015; 175(1): 454-68. [CrossRef]
24. Orhan H, Evli S, Dabanca MB, Başbülbül G, Uygun M, Uygun DA. Bacteria killer enzyme attached magnetic nanoparticles. Mater Sci Eng C Mater Biol Appl 2019; 94: 558-64. [CrossRef]
25. Kruger NJ. The Bradford method for protein quantitation. The protein protocols handbook, 2009; 17-24. [CrossRef]
26. Hilliard LR, Zhao X, Tan W. Immobilization of oligonucleotides onto silica nanoparticles for DNA hybridization studies. Anal Chim Acta, 2002; 470(1): 51-6. [CrossRef]
27. Betancor L, Fuentes M, Dellamora-Ortiz G, López-Gallego F, Hidalgo A, Alonso-Morales N, et al. Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles. J Mol Catal B: Enzym 2005; 32(3): 97-101. [CrossRef]

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ORIGINAL ARTICLE

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Hematopoietic Stem Cell Transplantation in Patients with Severe Combined Immunodeficiency: A Single-Center Experience

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ABSTRACT

Objective: The aim of this study was to determine the factors affecting outcomes in patients who underwent hematopoietic stem cell transplantation (HSCT) with the diagnosis of severe combined immunodeficiency (SCID). Furthermore, our aim is to share our single-center experience of HSCT among SCID patients.

Materials and Methods: The data of patients who underwent HSCT with the diagnosis of SCID between January 2014 and January 2021 in the pediatric bone marrow transplant unit of Istanbul Medipol University were retrospectively analyzed. Demographic and clinical data, treatment regimens, donor source, type of transplantation, pre- and post-transplantation infections, and complications were evaluated.

Results: Among fifteen patients who underwent HSCT, 5 (33%) were female. The mean age at diagnosis was 3 months (1-6 months), and at transplantation 6 months (3-10 months). The mean time from diagnosis to transplantation was 3 months (2-9 months). There was a history of consanguineous marriage in thirteen (87%) and sibling death in eight (53%) cases. As donors, six (40%) were siblings and five (33%) were unrelated, while four (27%) patients underwent haploid transplantation. Four (27%) patients died during the first 100 days of transplantation. The median follow-up period was 23 months (9-61 months). Overall survival probability was calculated as 73%.

Conclusion: SCID should be considered as an emergency in pediatrics. Devastating complications, including severe organ damage, life-threatening infections, and even death, could appear in case of diagnostic delay. HSCT is a currently available curative treatment option. Subjects with a confirmed diagnosis should be referred to the appropriate bone marrow transplant center and treated as soon as possible.

Keywords: Bone marrow transplantation, children, severe combined immunodeficiency

INTRODUCTION

Primary immunodeficiencies (PID) are defined by the inherited deficiency and/or dysfunction of components of the immunity. Today, more than 430 gene disorders have been reported to cause congenital defects of the immune system (1). Severe combined immunodeficiency (SCID) represents the most severe form of PIDs. SCID is a heterogeneous disease group caused by the development disorder and dysfunction of both T and B cells. It is divided into T-B- or T-B+ groups, according to the status of B lymphocytes. The frequency of PID in the community is estimated to be between 1/50000 and 100000 (2). In a study conducted in our country, it was reported as 1/10000 (3). While the X-linked form is more frequent in western countries, the autosomal recessive form is more commonly seen in our region. More than 18 gene disorders have been reported to cause SCID, so far.

Hematopoietic stem cell transplantation is the most efficient treatment option for patients with SCID, although

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several medical treatments and gene therapies have provided significant improvements in overall survival and quality of life for PID patients. Allogeneic hematopoietic stem cell transplantation (HSCT) has been performed for about 50 years in patients with PID, and the first successful bone marrow (BM) transplant was performed in 1968 (4).

Factors affecting prognosis include transplant age, pre-transplant infections and organ damage, human leukocyte antigen (HLA) compatibility, and preparation regimen. If the diagnosis is made in a timely way and HSCT is performed from a sibling with full HLA compatibility, the survival rate is over 90% (4).

MATERIALS AND METHODS

The file data of fifteen patients who underwent HSCT transplantation with the diagnosis of SCID between January 2014 and January 2021 in the pediatric BM transplant unit of Istanbul Medipol University were retrospectively analyzed. Diagnosis of the patients was established by laboratory evaluation (complete blood count, immunoglobulin (Ig) G, A, M levels, $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocyte, $CD19^+$ B lymphocyte and $CD16/56^+$ NK cell counts by flow cytometry) and confirmed by genetic testing. Age at diagnosis, transplantation age, gender, family history, HSCT type, donor type, stem cell source, preparatory regimen treatments, engraftment time and complications (pre- and post-transplantation infections), veno-occlusive disease (VOD), and graft versus host disease (GVHD) were recorded.

The local ethics committee of the hospital approved the study (June 2020-Decision no: E10840098). Written informed consent was obtained from the parents of all individuals.

Statistical Analysis

For statistical analysis, Statistical Package for Social Sciences (SPSS) package program V28.0 was used. For mean, median standard deviation data, continuous variables were used. Categorical variables were used for the frequency and percentage data. $p<0.05$ was considered significant.

RESULTS

Characteristics of the Patients

Of fifteen patients, five (33%) were girls and ten (67%) were boys. The median age of symptom onset was 2 months (1-3 months), at diagnosis 3 months (1-6 months), and at transplantation 6 months (3-10 months). The mean time from diagnosis to transplantation was 3 months (range: 2 and 9 months). The most common presentation findings were growth retardation and lower respiratory tract infection (LRTI). There was consanguinity among parents in thirteen (87%) patients, and a history of sibling death in eight (53%) patients. Clinical findings of the patients are shown in Table 1.

Table 1. Demographic and HSCT characteristics of the patients.

Patient No	Age at diagnosis (months)	Age at HSCT (months)	Sex	Consanguinity	SCID phenotype	Mutation	HSCT type	HLA compatibility	Stem cell source	Preparation regimen
1	2	5	M	Yes	T-B-NK+	*RAG1*	MSD	10/10	BM	None
2	3	6	F	Yes	T-B-NK-	*ADA*	MSD	10/10	BM	FLU/BU/ATG
3	1	3	M	Yes	T-B+NK-	*JAK3*	HAPLO	5/10	BM	FLU/BU/ATG
4	3	5	F	Yes	T-B-NK+	*RAG1*	HAPLO	5/10	BM	FLU/BU/ATG
5	6	9	F	Yes	T-B-NK+	*RAG1*	MUD	10/10	PBSC	None
6	5	9	M	Yes	T-B-NK-	*ADA*	MUD	10/10	BM	TREO/FLU/ATG
7	2	5	M	Yes	T-B-NK+	*RAG1*	MUD	10/10	UCB	None
8	1	3	M	Yes	T-B-NK-	*ADA*	HAPLO	5/10	PBSC	TREO/FLU/ATG
9	2	3	F	Yes	T-B-NK+	Unknown	MSD	10/10	BM	None
10	4	6	M	Yes	T-B+NK-	*FOXN1*	MSD	10/10	PBSC	TREO/FLU/ATG
11	3	7	M	No	T-B-NK+	Unknown	MUD	10/10	PBSC	TREO/FLU/ATG
12	5	9	F	Yes	T-B-NK+	Unknown	MUD	10/10	PBSC	TREO/FLU/ATG
13	4	10	M	No	T-B+NK-	*IL2RG*	MSD	10/10	BM	FLU/BU/ATG
14	2	6	M	Yes	T-B+NK+	Unknown	HAPLO	10/10	BM	TREO/FLU/ATG
15	4	8	M	Yes	T-B-NK-	*ADA*	MSD	10/10	BM	TREO/FLU/ATG

ADA: Adenosine-deaminase, ATG: Antithymocyte globulin, B: B cell, BM: Bone marrow, BU: Busulfan, F: Female, FLU: Fludarabine, FOXN1: Forkhead box protein N1 (FOXN1), HLA: Human leukocyte antigen, HSCT: Hematopoietic stem cell transplantation, IL2RG: Interleukin-2 receptor gamma, JAK3: Janus kinase 3 (JAK3), M: Male, MSD: Matched sibling donor, Haplo: Haploidentical, MUD: Matched unrelated donor, NK: Natural killer cell, PBSC: Peripheral blood stem cell, RAG1: Recombination activating gene 1, SCID: Severe combined immunodeficiency, T: T cell, Treo: Treosulfan, UCB: Umbilical cord blood.

Genetic analysis was performed in all the patients, and mutation was detected in eleven of them. There was no registered mutation in four patients. Adenosine-deaminase (*ADA*) mutations were found in four cases and recombination activating gene 1 (*RAG1*) mutations were registered in four cases. Interleukin-2 receptor gamma (*IL2RG*), forkhead box protein N1 (*FOXN1*), and janus kinase 3 (*JAK3*) mutations were recorded in one case.

Transplantation

As a donor source, six (40%) patients were transplanted from a fully matched sibling donor (MSD), five (33%) from a matched unrelated donor (MUD), and four (27%) from a haploidentical donor. BM samples were used in nine (60%) patients, while peripheral blood stem cell (PBSC) was used in five (33%) and cord blood in one patient as stem cell source.

While eleven (73%) cases were treated with reduced intensity conditioning (RIC) (intravenous treosulfan 14g/m^2/day, fludarabin 30 mg/m^2/day, busulfan 3.5 mg/kg/day, anti-thymocyte globulin 10 mg/kg/day), and prophylaxis for GVHD (cyclosporine A 3 mg/kg/day, methotrexate 10 mg/m^2), no regimen treatment was applied in four (27%) cases. Before transplantation, six (40%) cases had Cytomegalovirus (CMV) infection and four of them were intubated in the intensive care unit (ICU). Stem cells were applied to these cases in the ICU without regimen treatment. While three patients died during the first 28 days after transplantation, one patient died on the 45th day from sepsis and organ failure. The common features of deceased patients were poor general condition before transplantation and disseminated CMV infection. Bacillus Calmette–Guérin (BCG)

Table 2. Complications related to HSCT.

Patient No	Clinical features	BCG vaccination	BCG activation	Pre-HSCT infection	Pre-HSCT general condition	Acute GVHH	Outcome
1	Pneumonia, growth retardation	No	No	CMV	Poor, intubated	No	Fatal
2	Pneumonia, growth retardation	Yes	No		Good	No	Alive
3	Growth retardation, moniliasis	No	No		Good	Yes	Alive
4	Pneumonia, growth retardation	Yes	No		Good	Yes	Alive
5	Pneumonia, growth retardation, diarrhea	Yes	No	CMV	Poor, intubated	No	Fatal
6	Growth retardation, moniliasis	Yes	No		Good	No	Alive
7	Pneumonia, growth retardation, eczema	No	No	CMV	Poor, intubated	No	Fatal
8	Pneumonia, growth retardation, eczema	No	No		Good	Yes	Alive
9	Pneumonia	Yes	No	CMV	Poor, intubated	No	Fatal
10	Fever, diarrhea	Yes	Yes		Good	No	Alive
11	Growth retardation, moniliasis	No	No		Good	No	Alive
12	Pneumonia, growth retardation	No	No		Good	No	Alive
13	Pneumonia, growth retardation	Yes	No		Good	No	Alive
14	Pneumonia, growth retardation, eczema, moniliasis	No	No	CMV	Poor	Yes	Alive
15	Pneumonia, eczema	Yes	Yes	CMV	Good	No	Alive

BCG: Bacillus Calmette–Guérin, CMV: Cytomegalovirus, GVHD: Graft-versus-host disease, HSCT: Hematopoietic stem cell transplantation

vaccine was administered in seven (47%) cases. Anti-tuberculosis prophylaxis was applied to these cases during the transplantation process. BCG reaction developed only in one case.

While acute GVHD was developed in four (27%) patients who underwent transplantation from a haploidentical donor, chronic GVHD with chronic skin involvement developed in one patient. Lymphoproliferative disease and VOD did not develop in any patient. No patient required intravenous immunoglobulin (IVIG) infusion.

Engraftment took place in all patients, except for three patients who died during the first 28 days after transplantation. Eleven (73%) surviving cases are still being followed up in our clinic as fully chimeric. The overall survival probability was calculated as 73%.

DISCUSSION

SCID represents an urgent condition with fatal outcome in case of diagnostic and treatment delay. HSCT is the only available option for treatment of SCID patients (5,6). The aim of our study was to understand the factors affecting survival and mortality at the time of diagnosis in SCID patients who were treated with HSCT.

Early diagnosis significantly affects the survival of patients with SCID (7,8). Diagnosis is delayed in countries where newborn screening tests are not applied. Patients with suspected SCID should be referred to immunology specialists as soon as possible. If the diagnosis and treatment is delayed, severe organ damage and/or death can occur.

In some cases of partial SCID, such as Omenn's syndrome, immunologic laboratory markers could be observed in the range of normal values due to maternal engraftment, resulting in diagnostic delay (9,10). Two of our cases with Omenn syndrome (*RAG1* mutations) were late-diagnosed because the total lymphocyte count, lymphocyte subgroup, and immunoglobulin levels were found to be normal. CD45RO (memory T cell) marker should be checked in terms of maternal engraftment in patients with suspected SCID, with normal first-line immunological tests.

In general, positive family history leads to timely diagnosis. In a previous study, it was reported that patients with PID who were diagnosed at birth due to a positive family history were diagnosed earlier than their family members (11). In our study, 53% (n=8) of cases had a history of sibling death, and consequently they were diagnosed earlier than the patients without a family history.

One of the most important factors affecting survival in HSCT is early transplantation. Patients transplanted during the neonatal period (first 28 days of life) had better survival rates than those transplanted later (95% vs 76%; respectively) (12). T cell development was reported to be significantly improved in patients with HSCT in the first 3.5 months (13). The mean age of transplantation of our patients was six months.

Selection of a suitable donor for transplantation in patients is of critical importance. In general, stem cell source, conditioning treatment, and GVHD prophylaxis depend on the type of donor (14). HLA typing to evaluate potential donors should be performed in all family members immediately with the diagnosis of SCID. A sibling or related donor that is perfectly matched with HLA is preferred. When the patient does not have a suitable relative, an unrelated matched donor or haploidentical donor may be selected.

RIC therapy should be preferred in the pre-transplant regimen treatment. It is less toxic when compared to full myeloablative regimens. In one study, survival four years after HSCT was 94% in the RIC group versus 58% in the myeloablative conditioning group (15). A full myeloablative regimen was applied in none of our patients. Therefore, no side effects related to regimen therapy were observed.

Disseminated CMV infection was present in five (33%) cases before transplantation, and four of them died. Infections are responsible for most of the deaths that occur before or shortly after transplantation. While 5-year survival is 50% in infants with active infection, it is 82% in infants without infection (16). The most frequently detected organisms are CMV, Epstein-Barr virus (EBV), and adenovirus (17). Other important viral pathogens include respiratory syncytial virus, parainfluenza, enteroviruses, hepatitis viruses, and herpes simplex viruses (18). Administration of live vaccines to patients with SCID could cause severe infection (19). The live attenuated BCG vaccine causes disseminated mycobacterium infection. It is recommended to give anti-tuberculosis prophylaxis during the transplantation process to patients who have received the BCG vaccine. Live vaccines should not be administered in cases with a positive family history or suspected SCID (20). The polymerase chain reaction (PCR) method should be used in the diagnosis and follow-up of viral infections. Serological tests should not be used in the diagnosis because patients with SCID cannot form antibody responses and may have positive IgG titers reflecting maternal or exogenously administered IgG (21).

CONCLUSION

When the diagnosis and treatment of SCID is delayed, severe organ damage develops, or the patient dies from infections. Early diagnosis and transplantation in the early period have a significant positive effect on the survival rate. Patients should be screened for appropriate donors as soon as they are diagnosed and referred to an experienced transplant center for transplantation.

Ethics Committee Approval: Ethics committee approval was obtained from Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee. (June 2020-Decision no: E10840098)

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of Manuscript - S.N., S.A.; Final Approval and Accountability - S.N., Y.Y., M.E., N.B., K.Ö., A.K., S.A.

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

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REFERENCES

1. Bonilla FA, Khan DA, Ballas ZK, Chinen J, Frank MM, Hsu JT, et al. Practice parameter for the diagnosis and management of primary immunodeficiency. J Allergy Clin Immunol 2015; 136: 1186. [CrossRef]
2. Tangye SG, Al-Herz W, Bousfiha A, Chatila T, Cunningham-Rundles C, Etzioni A, Franco JL, et al. Human inborn errors of immunity: 2019 update on the classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol 2020; 40: 24. [CrossRef]
3. Kilic SS, Ozel M, Hafizoglu D, Karaca NE, Aksu G, Kutukculer N. The prevalences and patient characteristics of primary immunodeficiency diseases in Turkey-two centers study. J Clin Immunol 2013; 33(1): 74-83. [CrossRef]
4. Cowan MJ, Neven B, Cavazanna-Calvo M, Fischer A, Puck J. Hematopoietic stem cell transplantation for severe combined immunodeficiency diseases. Biol Blood Marrow Transplant 2008; 14(1 Suppl 1): 73-5. [CrossRef]
5. Lankester AC, Albert MH, Booth C, Gennery AR, Güngör T, Hönig M, et al. EBMT/ESID inborn errors working party guidelines for hematopoietic stem cell transplantation for inborn errors of immunity. Bone Marrow Transplant 2021; 56(9): 2052-62. [CrossRef]
6. Wahlstrom JT, Dvorak CC, Cowan MJ. Hematopoietic stem cell transplantation for severe combined immunodeficiency. Curr Pediatr Rep 2015; 3: 1. [CrossRef]
7. Miyamoto S, Umeda K, Kurata M, Yanagimachi M, Iguchi A, Sasahara Y, et al. Hematopoietic cell transplantation for severe combined immunodeficiency patients: a Japanese retrospective study. J Clin Immunol 2021; 41(8): 1865-77. [CrossRef]
8. Neven B, Leroy S, Decaluwe H, Le Deist F, Picard C, Moshous D, et.al. Long-term outcome after hematopoietic stem cell transplantation of a single-center cohort of 90 patients with severe combined immunodeficiency. Blood 2009; 113(17): 4114-24. [CrossRef]
9. Muller SM, Ege M, Pottharst A, Schulz AS, Schwarz K, Friedrich W. Transplacentally acquired maternal T lymphocytes in severe combined immunodeficiency: a study of 121 patients. Blood 2001; 98: 1847-51. [CrossRef]
10. Lev A, Simon AJ, Trakhtenbrot L, Goldstein I, Nagar M, Stepensky P, et al. Characterizing T cells in SCID patients presenting with reactive or residual T lymphocytes. Clin Dev Immunol 2012; 2012: 261470. [CrossRef]
11. Brown L, Xu-Bayford J, Allwood Z, Slatter M, Cant A, Davies EG, et al. Neonatal diagnosis of severe combined immunodeficiency leads to significantly improved survival outcome: the case for newborn screening. Blood 2011; 117: 3243. [CrossRef]
12. Griffith LM, Cowan MJ, Notarangelo LD, Kohn DB, Puck JM, Pai SY, et.al. Primary Immune Deficiency Treatment Consortium (PIDTC) report. J Allergy Clin Immunol 2014; 133(2): 335-47.
13. Myers LA, Patel DD, Puck JM, Buckley RH. Hematopoietic stem cell transplantation for severe combined immunodeficiency in the neonatal period leads to superior thymic output and improved survival. Blood 2002; 99: 872. [CrossRef]
14. Railey MD, Lokhnygina Y, Buckley RH. Long-term clinical outcome of patients with severe combined immunodeficiency who received related donor bone marrow transplants without pretransplant chemotherapy or post-transplant GVHD prophylaxis. J Pediatr 2009; 155: 834. [CrossRef]
15. Dvorak CC, Hassan A, Slatter MA, Hönig M, Lankester AC, Buckley RH, et al. Comparison of outcomes of hematopoietic stem cell transplantation without chemotherapy conditioning by using matched sibling and unrelated donors for treatment of severe combined immunodeficiency. J Allergy Clin Immunol 2014; 134: 935. [CrossRef]
16. King D, Davies GE, Gaspar HB, Veys PA. Improved survival after unrelated donor bone marrow transplantation in children with primary immunodeficiency using a reduced-intensity conditioning regimen. Blood 2005; 105(2): 879-85. [CrossRef]
17. Heimall J, Logan BR, Cowan MJ, Notarangelo LD, Griffith LM, Puck JM, et al. Immune reconstitution and survival of 100 SCID patients post-hematopoietic cell transplant: a PIDTC natural history study. Blood 2017; 130: 2718. [CrossRef]
18. Brown LK, Clark I, Brown JR, Breuer J, Lowe DM.. Norovirus infection in primary immune deficiency. Rev Med Virol 2017; 27: e1926. [CrossRef]
19. Patel NC, Hertel PM, Estes MK, de la Morena M, Petru AM, Noroski LM, et al. Vaccine-acquired rotavirus in infants with severe combined immunodeficiency. N Engl J Med 2010; 362: 314. [CrossRef]
20. Canessa C, Romano F, Lippi F, Bianchi L, Kashef S, Rezaei N, et al. Bcgitis and vaccine-derived poliovirus infection in a patient with a novel deletion in RAG1 binding site. Int J Immunopathol Pharmacol 2013; 26: 511. [CrossRef]
21. Dvorak CC, Puck JM, TePas E. Hematopoietic cell transplantation for severe combined immunodeficiencies. (Available from [updated 06/02/2022.;cited2022January].https://www.uptodate.com/contents/search/hematopoietic-cell-transplantation-for-severe-combined-immunodeficienciese).

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CASE REPORT

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Aspects of Psychological Stress in Success of Parathyroid Allotransplantation

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ABSTRACT

Parathyroid auto/allotransplant (PA) may be a valuable alternative in the treatment of permanent hypoparathyroidism which is defined by insufficient levels of parathyroid hormone (PTH). Here, we aimed to investigate a possible association between the PTH levels of the patients who had undergone PA and their psychological stress conditions. The three patients with hypoparathyroidism were reported before and after PA for three-month-follow-up period. The examinations of patients were assessed by biochemical parameters [intact PTH (iPTH), Ca, and P]. The anxiety and stress levels of patients were assessed by self-report measures: State-Trait Anxiety Inventory (STAI-S/T) and Perceived Stress Scale (PSS). Although iPTH levels of all patients had a tendency to increase after PA, one of the patients with a low level of anxiety and stress only reached the normal range of PTH levels. The recovery of the serum iPTH levels at month 1 post-PA was negatively correlated with PSS scores of patients ($r = -0.999$, $p = 0.028$). The outcomes of this report showed for the first time that insufficient PTH release, even after PA, might be related to the stress level of patients. By taking all results into consideration, PTH could be speculated as a stress biomarker.

Keywords: Parathyroid allotransplantation, parathyroid hormone, psychological stress, STAI, PSS

INTRODUCTION

Hypoparathyroidism is an uncommon endocrine disorder related to hypocalcemia, hyperphosphatemia, and low levels of parathyroid hormone (PTH) in the blood (1). The extended or complicated thyroidectomy operations often result in permanent hypoparathyroidism (2). Untreated hypoparathyroidism results in some complications such as nephropathy, cataract, muscle dysfunction, myositis, fasciitis, basal ganglia and/or cerebellar calcifications, and teeth malformations (3). Treatment with calcium and vitamin D replacement is recommended for deficient patients, yet this therapy may be inadequate (4).

Parathyroid allo/autotransplant is a valuable alternative strategy in treating hypoparathyroidism (5-8). Living patients or cadavers have been selected as donors in parathyroid transplantation. For many years, Aysan and his colleagues have been using new techniques to describe parathyroid allotransplantation (PA). According to one of their methods, after the mechanical disruption, the small pieces of parathyroid glands obtained from donors with parathyroid hyperplasia are grafted onto the patients with hypoparathyroidism (6). Another technique which is used by Goncu et al. (9) is a laparoscopic transplantation of cultivated parathyroid cell suspension into omentum rather than transplantation of tissue fragments.

During 12-month follow-up, success in the allograft function has been observed in most patients (8). However, increasing PTH levels after transplantation have suddenly declined in some of the patients. When the patients with altered PTH levels were questioned about their daily routines to investigate their psychosomatic problems, it was

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revealed that they had stressful/fatiguing events during those days. These conjectural findings turned our attention to discover the possible interaction between levels of PTH and psychological stress. The limited available information related to the levels of PTH and stress showed that patients with primary hyperparathyroidism were frequently associated with psychosis as well as with other psychiatric symptoms like dementia, anxiety, depression, lethargy or apathy, stupor, or coma (10). The current literature has also described secondary hypoparathyroidism accompanying mental disorders commonly with a picture of delirium, anxiety, or depression (11, 12). In a study, Tigranian and his colleagues found a remarkable increase in the PTH level, as well as an increase in blood pressure, in students before an examination (13). However, confirmatory evidence on the association between psychological stress and PTH remains insufficient to draw definitive conclusions at present. Therefore, we reported here, for the first time, the possible association between the PTH levels of three patients who had undergone PA and their psychological stress conditions.

CASE REPORT

This study describes three patients with chronic symptomatic hypocalcaemia and hypoparathyroidism who were admitted to our outpatient clinic. They all had a similar history that hypoparathyroidism emerged in the postoperative early stage after total thyroidectomy. The local human ethics commission of Bezmialem Vakif University approved the study, and informed consents were taken from the patients. Then, they were scheduled for PA. Following successful surgical interventions, all three patients were able to leave the hospital without any problems.

The three patients were assessed with the State-Trait Anxiety Inventory (STAI-S/T) (14) and Perceived Stress Scale (PSS) (15) tests. In a single session, patients completed both of the scales without assistance. There was no time limit, and all queries about the items were addressed. The STAI-S assesses a person`s current level of anxiety, whereas the STAI-T assesses a person`s long-term anxiety level and overall feeling. The STAI had a Cronbach's alpha reliability coefficient, ranging from 0.83 to 0.87 for STAI-S and from 0.26 to 0.68 for STAI-T. The STAI-S and STAI-T have scores ranging between 20 and 80. A higher test result suggests a higher level of anxiety. The three patients performed the Turkish version of both STAI-S and STAI-T (16) before PA and after PA for three-month follow-up period.

The PSS (a 14-item version) is the most validated psychological instrument for assessing stress perception. The perceived stress is a measure of stress based on stressful situations, and the ability to cope with them at an individual level over the previous month. The seven negative items in PSS are designated to examine a lack of control and unpleasant affective reactions, whereas another seven positive elements test the ability to cope with the current stressors. The ratings ranged from 0 to 56, with higher scores suggesting higher stress levels and the lower scores suggesting lower stress levels (17). The total score from the scale suggests the individual`s level of stress, indicating a low level between 11 and 26, a middle level between 27 and 41, and a high stress level between 42 and 56 (18). We demonstrated the occurrence of post-operation stress-related symptoms by assessing with the PSS at month 1 post-PA.

Statistical Package for Social Sciences (SPSS) 19.0 for Windows software was used for statistical analysis. Pearson's correlation test was performed to examine the relationship among the recovery of serum iPTH level and STAI-S, STAI-T, PSS scores at month 1 post-PA. $p \leq 0.05$ was considered for significant differences.

The serum PTH levels and the psychological conditions of three patients before and after PA were presented in Table 1. The iPTH levels of all patients were increased following PA. However, the levels of iPTH did not reach to the normal physiologic levels in the patients 1 and 3 (normal range for iPTH: 15 - 68.3 pg/mL). In contrast to the patients 1 and 3, the iPTH level of patient 2 reached to the normal range after PA. It was 10.7 pg/mL before PA, then increased to 16.4 pg/mL at month 1 after PA, 18.1 pg/mL at month 2 after PA, and 17 pg/mL at month 3 after PA. In addition, we did not notice any change for three-month follow-up period after PA in both calcium (normal range for Ca: 8.4 - 10.2 ng/mL) and phosphorus (normal range for P: 2.3 - 4.7 ng/mL) levels of patients were compared to the levels of those before PA.

Lastly, the STAI-S/T and PSS scores of patient 2 (STAI-S/STAI-T/PSS: 28/44/17) were lower than those of other two patients (STAI-S/STAI-T/PSS: 37/46/31 for patient 1; STAI-S/STAI-T/PSS: 51/57/43 for patient 3) at month 1 post-PA (Table 1). In addition, according to the results of Pearson's correlation test, the recovery of serum iPTH level at month 1 post-PA was negatively correlated with the PSS scores of patients ($r=-0.999$, $p=0.028$) (Table 2).

DISCUSSION

The role of the adrenergic system in the regulation of PTH secretion has not been clarified yet. However, it was observed that the secretion of PTH may be related to the presence of beta-adrenergic receptors and the sympathetic nerve endings on the parathyroid cells as a psychological stress response (19). The study began with observations in an outpatient clinic to determine which psychological risk factors were linked to alterations in PTH levels of patients who had undergone PA. We assumed that psychological factors and PTH levels had a causal relationship.

The effects of psychological stress on low graft function or rejection in organ transplantation are poorly understood, and this issue is valid for parathyroid gland transplantation, too. Most research on depression and/or anxiety looked at it pre- or early post- transplantation, and how it might affect post-transplant mortality risk. A growing body of evidence suggests that stress influences immune system that could lead to reduced lymphocyte function, especially in T-cell response to mitogens

Table 1. The results of three- month- follow-up for recipients.

		pre-PA	month 1 post-PA	month 2 post-PA	month 3 post-PA
Patient 1	**iPTH (pg/mL)**	10.3	13.1	13.9	13
	Ca (mg/dL)	8.5	7.5	7.3	7.3
	P (mg/dL)	5.7	5	5.6	5.3
	STAI-S	37	37	42	41
	STAI-T	46	46	51	49
	PSS		31		
Patient 2	**iPTH (pg/mL)**	10.7	16.4	18.1	17
	Ca (mg/dL)	8.4	7.4	7.8	7.2
	P (mg/dL)	4.1	4.1	5	4.8
	STAI-S	42	28	39	44
	STAI-T	36	44	48	43
	PSS		17		
Patient 3	**iPTH (pg/mL)**	10.4	10.3	11.7	12.6
	Ca (mg/dL)	7.3	7.7	6.7	7.3
	P (mg/dL)	4.7	4.5	5.6	5.7
	STAI-S	58	51	58	48
	STAI-T	61	57	54	57
	PSS		43		

iPTH: Intact parathyroid hormone; Ca: Calcium; P: Phosphorus; STAI: State and Trait Anxiety Scale; PSS: Perceived Scale Test; PA: Parathyroid allotransplantation.

Table 2. Statistical evaluation by Pearson`s correlation among the recovery of serum iPTH level (pg/mL) at month 1 post-PA and STAI-S, STAI-T, PSS scores.

Time	***month 1 post-PA***	
iPTH (pg/mL) correlations with	***r***	***p***
STAI-S	-0.992	0.079
STAI-T	-0.929	0.242
PSS	-0.999*	0.028

iPTH: Intact parathyroid hormone; PSS: Perceived Scale Test; PA: Parathyroid allotransplantation.*p< 0.05.

(20). In addition, depression appears to lead to higher C-reactive protein and pro-inflammatory cytokines levels (21), each of these factors raises the chance of death in the general population and in people with severe organ disease (22). Therefore, those aforementioned conditions might also adversely affect the adherence, and thereby undermine effective immunosuppression after organ transplantation. In an early study on 11 patients with acute rejection of a corneal graft which had occurred just after emotional stress, it was clearly evidenced that there was a relationship between psychological stress and graft rejection (23). Furthermore, depression was linked to a two-fold increased risk of graft failure, dialysis, and mortality with a working graft among kidney transplant patients (24).

In our study, all surgical interventions were successful due to the following factors: First, the patients were discharged from the hospital without any complications. In addition, they all had a tendency to increase their PTH levels after PA, too. Interestingly, the PTH level of patient 2 started to reach the normal range at month 1 post-PA and continued during the follow-up period. This patient had the lowest STAI-S and PSS scores implying low anxiety and stress level at the month 1 post-PA. Furthermore, we found that the recovery of iPTH levels of patients were negatively correlated with their perceived stress, rather than their anxiety levels at the month 1 post-PA. The findings of this study were in line with our previous animal study in which we discovered a negative connection between levels of iPTH

and corticosterone in acute restraint stress (25). In summary, all these observations suggested that there is a cross-connection between stress and PTH release.

CONCLUSION

This study concluded that PTH could be speculated as a stress biomarker. Thus, clinicians and psychiatrists should be alerted by the outcomes of this study to the possibility of stress as a risk factor for irregularity/alterations in the PTH levels among recipients after parathyroid transplantation.

Ethics Committee Approval: The local human ethics commission of Bezmialem Vakif University approved the study (15.09.2015-12926), and informed consents were taken from the patients.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - Ş.T.U., E.A.; Data Acquisition - Ş.T.U., E.A.; Data Analysis/Interpretation - Ş.T.U., B.E.; Ç.D.Ş.; Drafting Manuscript - Ş.T.U., B.E.; Critical Revision of Manuscript - B.E., Ç.D.Ş.; Final Approval and Accountability - Ş.T.U., B.E.

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

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REFERENCES

1. Aggarwal S, Kailash S, Sagar R, Tripathi M, Sreenivas V, Sharma R, et al. Neuropsychological dysfunction in idiopathic hypoparathyroidism and its relationship with intracranial calcification and serum total calcium. Eur J Endocrinol 2013; 168(6): 895-903. [CrossRef]
2. Testini M, Gurrado A, Lissidini G, Nacchiero M. Hypoparathyroidism after total thyroidectomy. Minerva Chir 2007; 62(5): 409-15.
3. Khan MI, Waguespack SG, Hu MI. Medical management of postsurgical hypoparathyroidism. Endoc Pract 2011; 17(Suppl 1): 18-25. [CrossRef]
4. Safioleas M, Stamatakos M, Rompoti N, Mouzopoulos G, Iannescu R, Salichou V, et al. Complications of thyroid surgery. Chirurgia (Bucur) 2006; 101: 571-81.
5. Nawrot I, Wozniewicz B, Tolloczko T, Sawicki A, Gòrski A, Chudziński W, et al. Allotransplantation of cultured parathyroid progenitor cells without immunosuppression: Clinical results. Transplantation 2007; 83: 734-40. [CrossRef]
6. Aysan E, Altug B, Ercan C, Kesgin Toka C, Idiz UO, Muslumanoglu M. Parathyroid allotransplant with a new technique: A prospective clinical trial. Exp Clin Transplant 2016; 14(4): 431-5.
7. Barczynski M, Golkowski F, Nawrot I. Parathyroid transplantation in thyroid surgery. Gland Surg 2017; 6: 530-6. [CrossRef]
8. Yucesan E, Goncu B, Basoglu H, Ozten Kandas N, Ersoy YE, Akbas F, et al. Fresh tissue parathyroid allotransplantation with short-term immunosuppression: 1-year follow-up. Clin Transplant 2017; 31(11). [CrossRef]
9. Goncu B, Yucesan E, Ozdemir B, Basoglu H, Kandas NO, Akbas F, et al. A new transport solution for parathyroid allotransplantation: Effects on cell viability and calcium-sensing receptors. Biopreser Biobank 2018; 16(4): 278-84. [CrossRef]
10. Geffken GR, Ward HE, Staab JP, Carmichael SL, Evans DL. Psychiatric morbidity in endocrine disorders. Psychiatr Clin North Am 1998; 21(2): 473-89. [CrossRef]
11. Ang AW, Ko SM, Tan CH. Calcium, magnesium, and psychotic symptoms in a girl with idiopathic hypoparathyroidism. Psychosom Med 1995; 57(3): 299-302. [CrossRef]
12. Rosa RG, Barros AJ, de Lima AR, Lorenzi W, Da Rosa RR, Zambonato KD, et al. Mood disorder as a manifestation of primary hypoparathyroidism: a case report. J Medical Case Rep 2014; 8: 326. [CrossRef]
13. Tigranian RA, Orloff LL, Kalita NF, Davydova NA, Pavlova EA. Changes of blood levels of several hormones, catecholamines, prostaglandins, electrolytes and cAMP in man during emotional stress. Endocrinol Exp 1980; 14(2): 101-12.
14. Spielberger CD. Manual for the state-trait anxiety inventory form y, self evaluation questionnaire. Palo Alto, CA: Consulting Psychologists Press, 1983. [CrossRef]
15. Cohen S, Kamarck T, Mermelstein R. A global measure of perceived stress. J Health Soc Behav 1983; 24: 385-96. [CrossRef]
16. Le Compte WA, Oner N. Development of the Turkish edition of the State-trait anxiety inventory. Cross-Cultural Anxiety 1976; 1: 51-67.
17. Mane Abhaya B, Krishnakuma, MK, Niranjan PC, Hiremath SG. Differences in perceived stress and its correlates among students in professional courses. J Clin Diagn Res 2011; 5(6): 1228-33.
18. Baltaş Z, Atakuman Y, Duman Y. Standardization of the perceived stress scale: Perceived stress in Turkish middle managers. Stress and Anxiety Research Society. 1998: In 19th International Conference, İstanbul.
19. Joborn H, Hjemdahl P, Wide L, Akerström G, Ljunghall S. Reduction of serum parathyroid hormone levels during sympathetic stimulation in man. J Endocrinol Invest 1987; 10(2): 153-6. [CrossRef]
20. Bartrop RW, Lazarus L, Luckhurst E, Kiloh LG. Depressed lymphocyte function after bereavement. Lancet 1987; I: 834-6. [CrossRef]
21. Elovainio M, Aalto AM, Kivimäki M, Pirkola S, Sundvall J, Lönnqvist J, Reunanen A. Depression and C-reactive protein: Population-based health 2000 study. Psychosom Med 2009; 71: 423-30. [CrossRef]
22. Tilg H, Wilmer A, Vogel W, Herold M, Nolchen B, Judmaier G, et al. Serum levels of cytokines in chronic liver disease. Gastroenterology 1992; 103: 264-74. [CrossRef]
23. Kok-van Alphen CC, Völker-Dieben HJ. Emotional stress and rejection, cause and effect. Doc Ophthalmol 1983; 56(1-2): 171-5. [CrossRef]
24. Dobbels F, Skeans MA, Snyder JJ, Tuomari AV, Maclean JR, Kasiske BL. Depressive disorder in renal transplantation: An analysis of medicare claims. Am J Kidney Dis 2008; 51: 819. [CrossRef]
25. Terzioglu-Usak S, Elibol B, Dallı T, Guler C, Aysan E. Effect of restraint stress on parathyroid hormone secretion in wistar rats. Int J Endocrinol Metabol 2018; 16(4): e66979.

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be

Table 1. Limitations for each manuscript type

Type of manuscript	Word limit	Abstract word limit	Reference limit	Table limit	Figure limit
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Review Article	5000	200	50	6	10 or total of 20 images
Case Report	1000	200	15	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	5	No tables	No media

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

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

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

References

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

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

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

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

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

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

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

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

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

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

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

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