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CONTENTS

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Potentiation of Cell Death and DNA Damage Through 5-Fluorouracil and Ferulic acid Coadministration in p53 Mutant HT-29 Cell Lines

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Abstract: When the Mediterranean diet is set in focus, scientific studies report a strong statistical correlation between human nutrition, diet, and cancer incidence. Considering the anticancer effects of a fiber-rich diet, it is understood that the anticancer effect is not only due to the bulk cellulosic material load but also related to the increased bioavailability of cellulose-bound bioactive (anticancer) compounds released due to intestinal microflora activities. Ferulic acid (FA) is one of the components found ubiquitously in the fiber fraction of plant food. Because of its effects on cancer cell viability and its association with a low incidence of cancer concerning a fiber-rich diet, FA can be considered an anticancer agent. In this work, it was investigated whether FA can potentiate the effects of anticancer drugs at lower doses. For this, a general anticancer drug named 5-Fluorouracil (5-FU) was used, and potentiation tests were performed on two cancer cell lines, namely A2780 besides HT-29, which has the homozygous mutation for p53. According to the results, it was interpreted that the anticancer effect of 5-FU was readily potentiated with 200 µM FA in both cancer cell lines, and DNA damage induced by 5-FU was potentiated by co-administration of FA. When cell viability and DNA damage of A2780 and HT-29 lines are evaluated together, we think it is most probable that 5-FU and FA administered jointly show its anticancer effect, especially by strengthening the apoptosis pathway triggered by DNA damage. If it might be possible to uncover the mechanism that drove DNA damage mediated apoptosis in p53 mutant HT-29 cells we may shed light on the treatment of chemotherapy-resistant cancer incidences. © 2023 NTMS.

Keywords: Cancer, HT-29; Chemotherapy; 5-Fluorouracil; Ferulic Acid.

1. Introduction

The famous Mediterranean Diet is a nutritional pyramid that rises on the shoulders of lightly to moderate cooked plant foods, fresh fruits, and vegetables. The second layer of this nutritional pyramid holds seafood and fish that provide saturated oils and minerals, while poultry, fermented products, meat products, and sweets make up the minor parts of this balanced diet. Various bioactive phytochemicals obtained from different plant sources have disease preventive effects, and some are proven to provide effective support in treatments against diseases such as atherosclerosis [1], thrombosis [2], cholesterolemia [3-5], diabetes [4-6], cancer [2, 5-7]. etc. On the contrary to Mediterranean Diet, an unbalanced diet progresses in

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the form of visceral fat, liver dysfunction, and obesity and results in various health problems [7]. Among these health problems, cancer cases hold a pretty significant fraction, and about one-third of cancer types are related to diet. In addition, it has been determined that there is a direct link between 13 types of cancer and obesity [8].
Although cancer therapy planning requires evaluation of cancer type, state of malignancy-metastasis, patient health status, age, etc., treatment is usually combined with chemotherapy and or radiotherapy due to lack of early diagnosis. In both chemotherapy and radiotherapy, the nontarget effects of treatment derive a general negative health status and exert negative pressure, especially on bodily essential organs like the liver, cardiovascular system, and kidneys [9-11]. The ultimate method of alleviating or even eliminating off-target effects in chemotherapy is targeted drug delivery [12, 13]. The basic idea behind targeted drug delivery stems from the problem that the dosage of the drug applied readily exceeds the tolerances of normal cells while barely killing cancer. A general pharmacologic trick in circumventing the overdosage of chemotherapeutics' is through drug interactions, i.e., administration with another bioactive compound and potentiating the anticancer effect at lower doses [14, 15].
5-Fluorouracil (Figure 1-A) is the reference cancer drug frequently used in cancer therapy studies where the context is determined as DNA damage and p53-apoptosis pathway [16, 17]. This drug interferes with nucleoside synthetic pathways and its metabolites are incorporated into nucleic acid chains during RNA and DNA synthesis, ending up with nucleic acid damage. One of its metabolites also complexes with Thymidylate Synthase (TS) and inactivates the enzyme, resulting in deoxy-thymidine depletion and consequently cytotoxicity and cell death [17]. 5-FU exerts its' cytotoxic effects on cancer cells through the tumor suppressor p53 regulated apoptosis pathway. The much-mentioned major disadvantage of this p53 apoptotic pathway approach is met with the apoptosis-resistant p53 mutant cancer cell lines. To eradicate cancer, drug combinations, dosage increment, or potentiating the 5-FU is usually considered at this stage.
Ferulic acid (Figure 1-B) is a phenolic acid, and it is described as one of the derivatives of cinnamic acid. This phenolic acid is a ubiquitous component of fiber material in the plant kingdom, and it is released in bulk amounts during processing of plant food [2, 18, 19]. This phenolic acid has low toxicity as its' glucuronic acid conjugates are readily absorbable and thoroughly excreted. The unconjugated portion of Ferulic acid is conjugated with glucuronide, and absorption can be achieved when the hindgut microbiota processes the fibrous plant parts. Ferulic acid is tested as a pharmacological agent in infection, inflammation, and cancer indications. It is frequently added to food and cosmetics products [2].

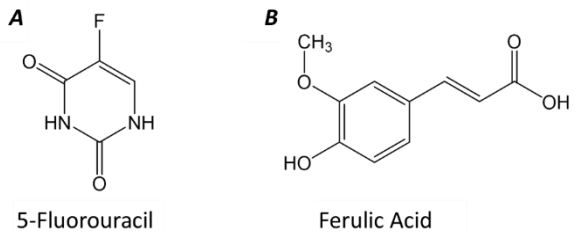

Figure 1. Chemical structure of *5-Fluorouracil* (A) and *Ferulic Acid* (B).

In this study Ferulic acid along with 5-Fluorouracil was applied on two cancer cell lines namely HT-29 and A2780 to test if 5-FU triggered p53 mediated apoptotic pathway could be potentiated at lower doses of 5-FU. Our aimed to test whether the anticancer effect of 5-Fluorouracil (a general anticancer drug) could be potentiated at lower doses on the HT-29 and A2780 cells when co-administered with Ferulic acid.

2. Material and Methods

2.1. Preparation of Cells Lines, Application of Ferulic Acid and 5-FU

HT-29 and *A2780* cell lines (ATCC, USA) were retrieved from liquid nitrogen and cell cultures were initiated in 75 cm^2 culture flasks with RPMI-1640 medium. Additives used in this media were 10 % *fetal bovine serum*, 1 % *penicillin*/ *streptomycin*, and 1 % non-essential amino acids. Cell cultures were maintained at 5 % CO_2 density at 37 °C throughout the experiment in Thermo Forma II CO_2 Incubator [USA]. After maintaining a proper confluency rate (min 90 %), cells were plucked out using *trypsin-EDTA* solution. Following the tests, cell vitality scores were measured with 0.4 % trypan blue. Cells with viability scores at least 90 % were used in the experiment. *5-FU* and *FA* test were started with a cell concentration of approximately 5×10^3 cells per well within 96-well plates. The test incubation durations were 24 hrs.
Working solutions of *Ferulic acid* and *5-FU* were prepared in DMSO. Cells were treated for 24 hours with different concentrations of compounds (25-400 μM for FU and 1-100 μM for 5-FU).

2.2. MTT Assay

MTT assay is the method of assessing cell viabilities through evaluation of colorimetric measure of metabolic activity. Measurements made in *MTT* assay depends on conversion of the tetrazolium salt (*MTT*: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to its insoluble purple colored formazan form. This conversion is handled with oxidoreductase enzymes in actively transpiring, metabolically active cells [20]. Highly condensed purple color designates cell viability whereas there exists no color development when cells are dead. Although *MTT* assay has many uses, it is utilized to test cytotoxicity of certain chemicals on cancer cells. In our work *MTT* assay was

performed as follows. First, whole liquid media was aspirated from the well, then 50 µl of 0.5 mg/ml *MTT* working solution was added into each well. After 3 hours of incubation at 37 °C in the incubator, *MTT* solution was aspirated out. Finally, 100 µl DMSO was added into the wells to release the *MTT* color developments, and scores were recorded in an *ELISA* reader (Thermo Multiscan Go, USA) at 570 nm wavelength [21].

The control group *MTT* scores (100 % viable) were taken as reference, and the test groups' viability scores were calculated concerning the control group [22, 23]. MTT scores were interpreted from at least cumulative of five independent experiment scores.

2.3. Alkaline Comet Assay

Following *5-Fluorouracil* (*5-FU*) and *Ferulic acid (FA*) applications, cancer cell lines' DNA damage tests were performed according to Alkaline Comet Assay (24). Depending on the initial viability test evaluations, concentrations of *5-FU* and *FA* used in Comet Assays were determined as 50 µM and 200 µM for *A2780* cells whereas it was determined as 20 µM and 200 µM for *HT-29* cells respectively. Test was performed on cells cultured in 6-well plates for 24 h culture period. First, whole media was aspirated, then cells were rinsed (2X) with PBS. Following this step, approximately 500 µl PBS was added to the well, and cells were scraped off the surface. Final cell suspension volume was adjusted to 1 ml with PBS.

10 µL of these suspended cells (approximately 10^4 cells/ µL) were added into 80 µL 1 % low melting agarose (LMA). The cell-LMA mix was overlaid on top of 1 % standard agarose-coated slides, and the surface leveling was done with lamella laid on top of the microscopy slides. Following cooling and gel solidification lamella were gently removed and cell lysis were performed. For lysis, slides were treated in lysis solution for 1 h at 4 °C. Lysis solution composition was 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton-X, and 10 % DMSO where pH 10 was maintained. Following lysis slides were electrophoresed at 25 V (5V/ cm, max. 300 mA) for 30 min. Finally, the slides were washed in neutralization solution three times for 5 min; neutralization solution was composed of 0.4 M Tris at pH 7.5. Staining was performed with ethidium bromide and the DNA damages were examined under fluorescence microscope (Zeiss Axio-scope, Germany). Tail DNA (%), evaluations were performed on randomly selected 250 cells from each group and performed with TriTek Comet Score software.

2.4. Statistical Analysis

Statistical analyzes were performed with Sigma Plot 12 package program. Data were interpreted as percentiles of mean values and standard deviation scores (Mean±SD). Following normality and homogeneity analysis, data variances and intergroup mean-variance comparisons were made with the Kruskal-Wallis H test. Comparisons of Comet Assay group data were performed with Mann-Whitney U test. Scores that fall into the $P<0.05$ probability domain were considered significant.

3. Results

3.1. Viabilities of A2780 Cell Lines with respect to 5-FU and FA Dosages

Viability tests of the human ovarian cancer cell line (*A2780*) treated with 100 µM *5-FU* showed a significant reduction in viability scores (around 12 %) compared to the control groups (Figure 2-A). Besides this, *A2780* cell line viabilities were also tested against different concentrations of *Ferulic acid (FA*) (25-400 µM) (Figure 2-B). The effective dose that scored significantly lower viability rates (around 18 %) was determined as 200 µM FA. Based on these results, in *5-FU* potentiation tests, the *5-FU* concentration was fixed at 50 µM, while the *FA* concentration ranged from 50 to 400 µM (Figure 2-C).

Compared with *FA* data (given in Figure 2-B), administration of 50 µM *5-FU* combined with 200 µM *FA* decreased *A2780* cell viability from 18 % to 34 % (Figure 2-C). Similarly, in the presence of *5-FU*, when the *FA* concentration was shifted to 400 µM, *A2780* cell viability was decreased from 51% (Figure 2-B/ 400 µM *FA* data) to 70 % (Figure 2-C/ 400 *FA* and 50 µM *5-FU* data).

As a result, it was observed that the drug interaction worked and the viability of *A2780* cells decreased from 66 % to 30 % when the *FA* dose was increased from 200 µM to 400 µM in *A2780* cells treated with *5-FU* (Figure 2-C).

3.1. Viabilities of HT-29 Cell Lines with Respect to 5-FU and FA Dosages

Viability tests on *HT-29* cell lines treated with both 50 and 100 µM *5-FU* showed statistically significantly reduced viability (approximately 12 %) when compared to control groups (Figure 3-A). Along with it, viability tests performed at varying concentrations of *Ferulic acid (FA*; 25-400 µM) revealed that *HT-29* cell line viabilities dropped more at 200 and 400 µM *FA* (Figure 3-B) as compared to that of *A2780* (Figure 2-B). Compared to 18 % and 51 % decrease in cell viabilities of *A2780*, *HT-29* cell line viability decreases were scored as 31 % and 62 % at 200 and 400 µM *FA* concentrations applied respectively. That is, *FA* showed a more substantial effect in *HT-29* cell lines compared to *A2780*. According to the results obtained from the experiments performed on *5-FU* and *FA* separately with *HT-29* cell line, in the *5-FU* potentiation experiments, *5-FU* concentration was fixed to 25 µM while the *FA* was changed from 50 to 400 µM (Figure 3-C).

When *5-FU* and *FA* were applied together, it was observed that *HT-29* cell death progressed from about 46 % to 66 % as the *FA* concentration was increased from 200 to 400 µM. When whole viability tests of *HT-29* were compared, it was recorded that 25 µM *5-FU*

resulted in 5 % cell death and, when 200 μM *FA* is used, 32 % cell death occurred, and finally when 25 μM *5-FU* and 200 μM *FA* are used together, 45 % cell death rate is observed. As compared with the test results of the *A2780* cell line, these above-stated cell death scores for the *HT-29* cell line were higher with the combinatorial use of *5-FU+FA* than only *FA* use.

3.3. DNA Damage Scores of A2780 and HT-29 Cell Lines with 5-FU and FA Combination

Depending on the data presented above for previous experiments, it was observed that the anticancer activity of 5-FU at low doses can be potentiated when coupled with FA. Based on these results, the concentrations to be applied in combinatorial use experiments were determined as follows; the FA was fixed at 200 μM, while the concentration of 5-FU was set at 20 μM and 50 μM for the HT-29 and A2780 cell lines, respectively. DNA damages in cells exposed to these fixed dosages were determined by Alkaline Comet Assay.

When the *Comet Assay* results of control for both cell lines were compared, it was observed that the *A2780* cell line containing wild-type p53 produced approximately 5.5 % tail DNA (DNA damage score of the median), while the *HT-29* cell line homozygous for the p53 mutation produced 22 % tail DNA (DNA damage score of the median). This case can be explained as follows: In the *A2780* cell line with wild-type p53, cell-cycle is arrested till DNA damage repair is achieved, whereas in the p53 homozygous mutant cell line *HT-29*, cell cycle checkpoints are bypassed, and DNA damage is accumulated due to the nonfunctional (mutant) p53 copies (Figure 4-A and B control groups compared).

When only *5-FU* (50 μM) is applied to *A2780*, group median (cells) accumulated 57 % Tail-DNA (damage) while, *5-FU* (50 μM) and *FA* (200 μM) co-administration resulted in accumulation of 67 % Tail-DNA in the group median cells (Figure 4-A). When both groups' quantile and the median DNA damage scores are compared, we have seen that the Q3 (3rd quantile) DNA damage squeezed to 69.5 % with *5-FU* application is jumped to 74 % if *5-FU* and *FA* applied together. That is as we evaluated both *A2780* cell viability (Figure 2-C) and DNA damage (Figure 4-A) although co-administration of *5-FU* and *FA* enhances cell death (to 34 %), DNA damage potentiation rate increased around 5%. In other words, *5-FU* and *FA* potentiated *A2780* cell death might not be related to DNA-damage and apoptosis.

On the other hand, if only *5-FU* (20 μM) is applied to *HT-29* cell line, the group median accumulated 29 % (Tail-)DNA damage but quantile range distribution pattern resembled that of the control. As an argument to support this we can state that, with the *5-FU* administered group, second quantiles' DNA damage scores were more condensed compared to the third quantile scores (Figure 4-B). Compared to 29 % DNA damage score of only *5-FU* administration, *5-FU* and *FA* co-administration on *HT-29* resulted a median DNA damage score of 46 %. And, in the second and third quantile range this data showed almost normal distribution. Depending on the overall evaluation of both viability and DNA damage data for *HT-29*, we may postulate that, *5-FU* and *FA* co-administration potentiated both *HT-29* cell death and DNA damage.

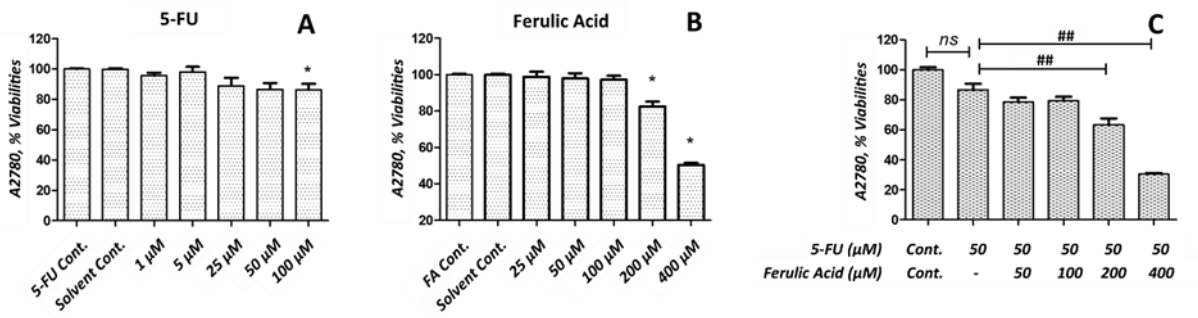

Figure 2. Cell viability tests performed on A2780 cell lines with 5-Fluororuracil (A), with Ferulic acid (B) and with 5-FU+FA combination. Data represented are mean scores capitated with standard deviation calculations. *p<0.05 vs control, ## p<0.01 between groups; ns: non-significant.

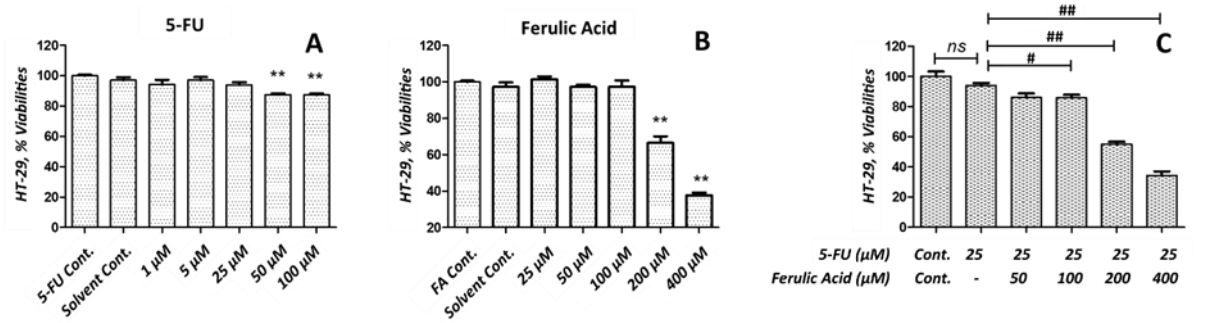

Figure 3. Cell viability tests performed on HT-29 cell lines with 5-Fluororuracil (A), with Ferulic acid (B) and with 5-FU+FA combination. Data represented are mean scores capitated with standard deviation calculations. ** p<0.01 vs control; # p<0.05 and ## p<0.01 and ### between groups; ns: non-significant.

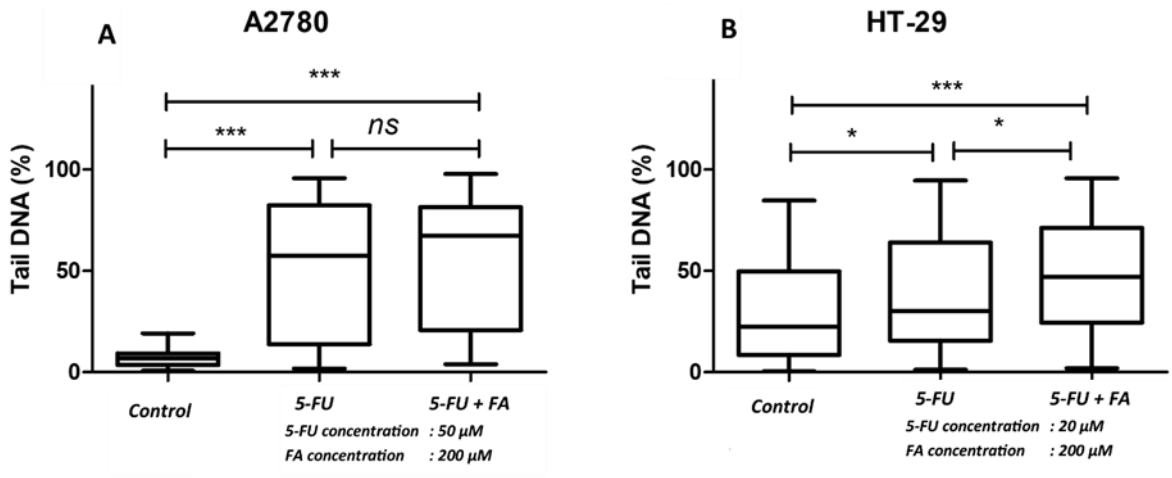

Figure 4. DNA damage data were interpreted as whisker-box plots. DNA damage score of the sample median was denoted with horizontal transverse lines in each box. The Mann-Whitney U test was applied for comparisons between groups. *p<0.05, **p<0.01, ***p<0.001.

4. Discussion

Colorectal cancer is among the most prevalent cancer incidence seen worldwide with both sexes and usually progresses into malignant states before diagnosis [25]. In relation to in vitro anticancer studies performed on colorectal cancer, HT-29 is generally the preferred cell line. In association with our experimental design, since our aim was to potentiate 5-FU at lower dosages we have preferred HT-29 cell line that possesses homozygous p53 mutation characterized as Arginine 273 to Histidine conversion and also shows a level up (mutant) p53 expression to a higher titer [2, 25, 26].

A2780 is the ovarian cancer cell line which had been recovered from an untreated patient; that is, this cell line was not exposed to any anticancer drug or chemical. Therefore, it is in demand as a cell line for testing potential anticancer chemicals and different drug delivery methods [27]. Also it is important to note that A2780 expresses wild type p53 [28]. In this study, together with the HT-29 cell line (p53 mutant), A2780 was used as the reference (control) cancer cell line expressing wild-type p53 during testing 5-FU drug potentiation with Ferulic acid.

Depending on the test results evaluated, we may state that administration of 5-FU coupled to FA decreased the cell viabilities of both A2780 (wild type p53) and HT-29 (homozygous mutant for p53) cancer cell lines. And we can also state that in 5-FU+FA administered HT-29 cell lines, DNA damage was normalized and enhanced by insignificant rates compared to the A2780 groups.

In the scientific literature Ferulic acid had been tested for its supportive function in alleviating multidrug resistance in cancer cell lines. FA was shown to bind P-glycoprotein and inhibit excretion anticancer drugs [29]. Works on multidrug resistance phenomenon point out indirect downregulation of mdr1b by FA [30]. Although in some other FA-anticancer evaluations, cancer cell death had been proposed as un-linked to p53 apoptotic pathway [31], reports also point out FA and Cu interaction and consequently ROS generation and DNA damage as putative cancer therapy treatment [32]. Still some other reports point out FA as potent anticancer agents triggering cell cycle stall and autophagy in cancer cells [33]. Depending on the literature although one can declare that anticancer effect of FA might not be through p53 mediated apoptosis, it is obvious that FA exerts its anticancer effects thorough multiple routes.

5. Conclusion

Depending on the literature reviews and our observations we can state that FA and 5-FU co-administration may induce cell death with different routes in A2780 and HT-29 Cell lines. It should still be questioned if FA may or may not be triggering p53 mediated apoptosis in wild type p53 A2780 and mutant p53 HT-29 cell lines as co-administered with 5-FU. Our observation also is in concert with the finding that postulates FA inhibiting or hampering multidrug resistance phenotype (Figures 2 and 3). It was also interesting to find out that 5-FU+FA co-administration stacked DNA damage of the third quantile (cells) to higher rates in both A2780 and HT-29 cells (Figure 4). This data is also in concert with the information given in the report of Sarwar et al. [32].

Depending on the experimental data and literature reviews we may conclude that, since FA has selective potential on cancer cells and has direct impact in DNA integrity [33] while it also potentiates the effects of other anticancer drugs like 5-FU, further diagnosis on combinatorial use of FA with other drugs may point out plausible cancer chemotherapies along with revealing alternative apoptotic mechanisms as models for cancer regression.

Limitations of the Study

Within the scope of the study and experimental design, the author cites the apparent shortcoming of the study as the inability to test the dissolved effective concentrations of the tested chemicals.

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Conflict of Interests

Author declares no conflict of interest with this study and manuscript.

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Author Contributions

The entire text of the work is attributed to the responsible author.

Ethical Approval

Scientific works undertaken with this study did not require any ethical approval.

Data sharing statement

Author declares; All data generated by this work are publicly available as long as reference rights are not violated if any part of the work is used.

Consent to participate

Author declares; There are no other participants related to this work.

Informed Consent

Author declares; Since the work is self-contained, there is no need of another researchers
consent approval.

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Evaluation of Patients Presenting to the Emergency Department with Suicidal Drug Poisoning

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Abstract: Intoxications constitute 1-2 % of emergency service applications. The most common cause of poisoning is suicide attempts. Of these attempts, 95 % are done by using drugs. We aimed to contribute to the literature by investigating the diagnosis, treatment, prognosis, and demographic data of patients admitted to the emergency department due to drug intoxication. Patients over 18 with complete data on patient files and the hospital automation system between October 1st, 2017 and October 1st, 2018 were included in the study, whereas patients under 18 years of age and those with incomplete data were excluded. A total of 126 patients presented to the emergency department with drug poisoning. The mean age of patients was 33.58±13.58 years. The female-male ratio was determined as 1.93. One out of 126 patients was intubated and referred to the intensive care unit (ICU). Seventy-six of the admitted patients (60 %) were hospitalized for inpatient treatment and follow-up, while 49 (39 %) were cared in the emergency department. Multiple drugs were the most common cause of poisoning. The highest time interval for drug-intoxicated emergency department admissions was between 18:00 and 24:00. Of the patients, 86 were previously treated for a psychiatric illness. The psychiatric consultation rate was significantly higher in hospitalized patients. Admissions to drug intoxication clinics due to suicide attempts are predominantly composed of women. Application hours are generally during the intensive admission periods of the emergency department. Arrangements should be made for both general medical care and psychiatric treatment of suicidal drug poisoning cases in emergency service applications. © 2023 NTMS.

Keywords: Healthcare Professionals; Anxiety; Hospital Support Staff; Pandemic.

1. Introduction

Drug intoxications may be associated with accidental or suicidal ingestion of toxic agents. In both cases, emergency treatment includes administration of the relevant antidote and gastric decontamination. If necessary, patients are followed up in wards and intensive care units [1].

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Studies in the United States have reported 2.3 million cases of acute intoxication per year [2-4]. In Turkey, the annual number of patients admitted due to acute intoxications is estimated at around 150,000 [5]. Admission rates due to intoxication vary between 0.46 % and 1.57 % among all emergency departments [6]. Although intoxications come with different agents, they are primarily associated with the use of medical drugs [1].

In this study, we aimed to evaluate the demographic and clinical prognoses of patients who applied to the emergency department with drug intoxication.

2. Material and Methods

The study was conducted with retrospective data collected from patients admitted to our emergency department due to drug intoxication between October 1st, 2017 and October 1st, 2018, a hospital with an average 600 daily and 220,000 annual admissions.

Ethics committee approval was obtained from the ethics committee of Çanakkale Onsekiz Mart University (numbered 2019/02, dated 16/01/2019).

We included patients over 18 who had complete data in patient files and the hospital automation system between October 1st, 2017 and October 1st, 2018.

The patients' data were collected from the hospital automation system and patient files, and were recorded on a data collection form.

The demographic characteristics, admission time and history associated with psychiatric diseases of patients who applied to the emergency department with drug intoxication were extracted.

2.1. Statistical Analysis

Statistical analysis was performed using SPSS 23.0 for Windows® statistical program (IBM Inc. Chicago, IL, USA). Continuous variables with normal distribution were expressed as mean ± standard deviation (SD), skewed data were reported as median (minimum-maximum), and categorical variables were described as numbers and percentages. For continuous variables, Mann–Whitney U-test was used in the groups with skewed distribution, whereas Student's t-test was used in the groups with normal distribution to determine the significance between the group means. Pearson's Chi-Square and Fisher's Exact Test were used to test the significance between categorical variables. All p-values were reported as two ways. A p-value of <0.05 was considered significant.

3. Results

A total of 225,859 patients were admitted to the emergency department between October 1st, 2017 and October 1st, 2018. Of these, 126 patients presented to the emergency department with drug poisoning. The mean age of patients was 34±14 years ranging from 18 to 92. There were 83 females with a female/male ratio of 1.93. One out of 126 patients was intubated and referred to the intensive care unit (ICU) and died.

Seventy-six of the admitted patients (6 0%) were hospitalized for inpatient treatment and follow-up, while 49 (39 %) were cared in the emergency department. One patient left the inpatient service and two the emergency department without doctors' approval. Multiple drugs had the highest frequency among causes of drug intoxication, whereas paracetamol and other analgesics were identified as the active substance and the drug groups, respectively, with the highest frequency in the single drug intoxications (Table 1).

Table 1: Frequency of drug groups.

	Number (n)	Percent (%)
Multiple drugs	53	42.1
Analgesics	31	24.6
Antidepressants	13	10.3
Antibiotics	11	8.7
Antipsychotics	5	3.9
Other	13	10.3

Drug poisonings occurred most frequently in November. The drug intoxication frequency was significantly higher in winter than summer (Figure 1).

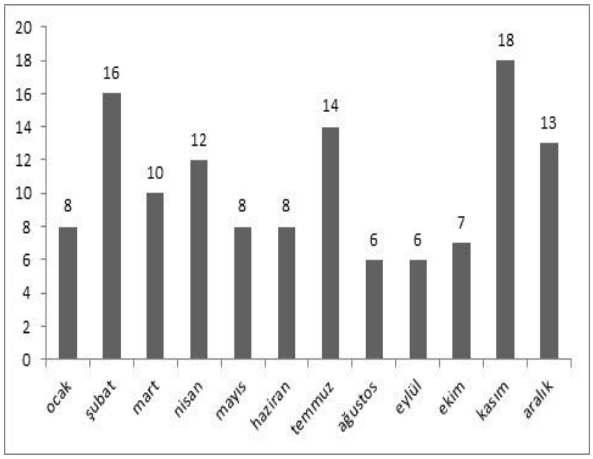

Figure 1: Distribution of the number of cases by months.

The highest application time interval was the six hours between 18:00-24:00 at a rate of 61% (Figure 2).

The patients were examined in terms of psychiatric disease histories. Eighty-six of 126 patients were previously treated for a psychiatric illness (68 %). Eighty-two patients had abnormal psychiatric findings and received psychiatric consultation. The rate of requesting psychiatric consultation was significantly higher in patients with a history of a psychiatric illness than in those without ($p = 0.015$) The rate of psychiatric consultation in hospitalized patients was significantly higher than discharges from the emergency department ($p < 0.001$).

Recurrent emergency service applications within 24 hours of patients who applied to the emergency department with drug intoxication were examined. The rate of recurrent hospital admissions was higher in patients discharged without consulting the psychiatry department (60%), with a statistically insignificant difference (p = 0.083) (Table 2).

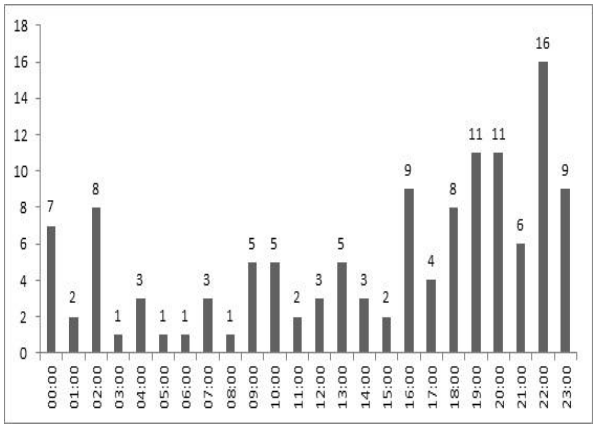

Figure 2: Distribution of the number of cases according to the hours of admissions.

Table 2: Comparison of patients consulted and not consulted to the psychiatry department.

		Patients consulted to the psychiatry department (n: 82)	Patients not consulted to the psychiatry department (n: 44)	
	Age	35.29±14.46	30.39±11.24	0.045
Gender	Female (n = 83)	52 (62.7 %)	31 (37.3 %)	0.427
	Male (n = 43)	30 (69.8 %)	13 (31.2 %)	
History of a Psychiatric Treatment	Yes (n: 86)	62 (72.1 %)	24 (27.9 %)	0.015
	No (n = 40)	20 (50.0 %)	20 (50.0 %)	
Hospitalization in any Department	Yes (n = 77)	62 (80.5 %)	15 (19.5 %)	<0.001
	No (n = 49)	20 (40.8 %)	29 (59.2 %)	
Recurrent Admissions	Yes (n = 10)	4 (40.0 %)	6 (60.0 %)	0.083
	No (n = 116)	78 (15.5 %)	38 (84.5 %)	

4. Discussion

As in all the world, the frequency of suicide attempts and suicide deaths are increasing every year in Turkey. International literature has reported that the most common methods of suicide attempts resulting in death include hanging and firearms in males, whereas drug/substance intake in females [7-10]. The literature studies reported a higher number of females than males involved in suicide attempts [6, 11-14]. The present study reported a higher rate of female gender (65.9 %) among cases of intoxication than that of male gender.

Literature studies have reported a mean age range of 25 to 30 years. In our study, the mean age was 33.58±13.58 years, which was higher than in the literature studies [6, 13-16].

In various countries, intoxications have been associated with different agents, most often with the use of medical drugs.1 In Turkey, antidepressants and analgesics usually rank first among the agents causing intoxication [6, 11, 12, 15, 17]. Drug intoxications may be associated with the ingestion of a single drug, as well as of multiple drug [5, 14, 18]. In our study, in accordance with other studies, drug intoxications were most commonly associated with ingestion of multiple drugs. In addition, analgesic agents ranked first among the drug groups causing intoxication.

In the present study, the majority of cases of intoxication were admitted to the emergency department in the winter months. In contrast to our study, studies conducted in Turkey [19-21] and abroad [22, 23]. examining the seasonal distribution of patients admitted due to intoxication, reported the highest frequency of patient admissions in the spring and summer months.

In a study conducted in Turkey, 64.4 % of patients were transferred to the ward and 33.3 % were discharged with recovery without complication [15]. The rate of inpatients due to acute intoxications in intensive care units varies between 3.4 and 13.8 % [24, 25]. In our study, of all patients, 76 (60.3 %) were hospitalized in the ward and 1 (0.8 %) in the intensive care unit, while 49 (38.9 %) were treated in the emergency department.

In our study, 1 patient (0.8 %) had mortality due to intoxication. In addition, Taş et al. reported a mortality rate of 0 % [26]. This rate was 0.92 %, 0.31 % and 10 % in the studies of Özayar et al. [6] and Yağan et al. [27] respectively.

In our study, 68.3 % of the patients had a history of a psychiatric disease and 7.9 % had a history of a similar suicide attempt. In a study conducted in Turkey, 35.3 % of the patients had a history of a psychiatric disorder [12].

Psychosocial status of the patients can be neglected when trying to provide the best possible medical care in emergency departments. Another shortcoming is the lack of referral to the necessary psychosocial support following medical treatment for suicide attempts in emergency departments. A timely psychotherapeutic intervention following medical care is an approach that provides easy and quick beneficial results in these patients. Some studies have reported that 7 to 10 % of adolescents have a history of a suicide attempt, of which about 2 to 3 % received medical care, and less than 50 % were referred to psychotherapy following medical care in emergency departments, of which the majority discontinued psychotherapy [28]. Therefore, it is also important to perform psychiatric evaluation of patients admitted to the emergency department due to a known suicide attempt. In our study, 34.9 % of the patients were not consulted to the psychiatry department.

An admission to the emergency department following an incomplete suicide attempt has been reported to be an important factor that increases the likelihood of a subsequent fatal suicide attempt [29]. In this respect, suicide attempts are more important than completed suicides. This is because 10 to 20 times more suicide attempts occur for each completed suicide.10 Not only those who have a psychiatric problem, but also those who are defined as 'normal' in psychiatric terms may have suicidal behavior [10]. In our study, 7.9 % of the patients attempted suicide again within a year.

Drug intoxications continue to be a growing problem for physicians working in emergency departments in Turkey as well as in the world. The incidence of drug intoxications was higher in patients who had previously applied to psychiatry outpatient clinics than those who had not. Our study highlighted the shortcomings of physicians in emergency departments in referring this patient group to the psychiatry department. Adequate psychosocial support is important in patients who come to prevent recurrent suicide attempts.

5. Conclusions

Women are remarkably high in attempted suicide cases admitted to the emergency department with drug intoxication. Application hours are during intensive admission hours of emergency departments. Arrangements should be made for both general medical care and psychiatric treatment of suicidal drug poisoning cases in emergency services.

Limitations of the Study

Our study has some limitations, including a regional retrospective study design, a relatively low number of patients, and a one-year follow-up for recurrent admissions.

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Conflict of Interests

The authors declare no conflict of interest.

Financial Support

The authors have no commercial associations or sources of support that might pose a conflict of interest.

Author Contributions

Conceived and designed the experiments; B.V., C.A., S.Y., K.A, O.Ç. Analyzed and interpreted the data; B.V., K.A, O.Ç. Contributed reagents, materials, analysis tools or data; B.V., C.A, S.Y., K.A, O.Ç. Wrote the paper; B.V., C.A, S.Y.

Ethical Approval

Ethics committee approval was obtained from the local ethics committee of the tertiary health center (Degree date/no: 16.01.2019/ 2019-02).

Data sharing statement

Data and statistical analysis plan will be shared if requested.

Consent to participate

Consent was obtained from all patients for the use of data under ethical conditions.

Informed Statement

Informed consent forms were obtained from all patients the patient data could be used in the retrospective studies.

Presentation(s) or Awards at a Meeting

Oral Presentation (I. Ulusal Acil Tıpta Toksikoloji Sempozyumu, İstanbul, 14-15 December 2018).

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Vestibular Evoked Myogenic Potential Abnormalities in Early and Late-Stage Parkinson Patients

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Abstract: Loss of balance can be seen in idiopathic Parkinson's disease. There are only a few studies in the literature in which brainstem involvement in IPD has been researched with neurophysiological tests such as vestibular evoked myogenic potential. In this study, it was investigated whether there is a difference in the results of vestibular evoked myogenic potential testing in early or late stage of idiopathic Parkinson's disease. The idiopathic Parkinson's disease cases were classified as early stage and late stage according to the Hoehn-Yahr scale. The presence of a positive wave with a latency of P13 and a negative wave with a latency of N23 was investigated as the first reflex response The latencies of these potentials and the absolute amplitude of the P13-N23 component were measured. The vestibular evoked myogenic potential results of the patients with early and late stage idiopathic Parkinson's disease were compared with those of the control group. The right P13 latency mean value in the late-stage patient group was significantly prolonged than in the early-stage patient group and the control group. The right P13-N23 amplitude mean value of the late and early-stage patient groups was significantly smaller than that of the control group ($p < 0.002$ and $p < 0.001$, respectively). Among the patients with idiopathic Parkinson's disease, the P13 latency was statistically increased in those with a fall history than in those without a fall history. As a result, this study indicates that the vestibular evoked myogenic potential pathway is affected over time especially in patients with late-stage Parkinson's disease. © 2023 NTMS.

Keywords: Parkinson; c VEMP; Neurophysiology; Brainstem.

1. Introduction

Parkinsonism is a chronic, progressive neurodegenerative disease characterized by restingtremor, rigidity, bradykinesia, and postural instability. The most common cause of parkinsonism is idiopathic Parkinson's disease. The main pathological changes in Parkinson's disease are the loss of melanin-containing dopaminergic neurons in the substantia nigra pars compacta (SNc) and the presence of Lewy bodies (LB) in surviving neurons, and Lewy neurites in axons. Immunohistochemical staining data show that Lewy bodies predominantly contain alpha-synuclein, ubiquitin, neurofilaments and many other different

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proteins [1]. The decrease in dopaminergic activity because of the degeneration occurring in dopaminergic neurons in the nigrostriatal pathway is the main cause of the disease. In a pathological study based on the accumulation of alpha-synuclein, Braak et al. asserted that the pathological process began in the olfactory bulb, caudal brainstem structures and even in the cardiac and gastrointestinal peripheral autonomic system long before the SN, comprised serotonergic, cholinergic, and noradrenergic neurons in the locus coeruleus, median raphe and nucleus basalis, and that widespread cortical involvement occurred in the progressive process [2, 3]. Clinical motor symptoms in IPD appear following a pre-clinical period of 4-6 years during which approximately 60 % of nigral dopaminergic neurons are lost [4]. In addition, motor symptoms, non-motor symptoms including depression, dementia, anxiety, psychosis, sleep disorders, autonomic dysfunction (symptomatic orthostatic hypotension, erectile dysfunction, micturition-defecation problems) are common in IPD, and many of these symptoms may also develop before the onset of motor symptoms. Parkinson's disease is considered to be the second most common neurodegenerative disorder after Alzheimer disease [4, 5]. As treatments that modify and perhaps prevent the disease become available, the importance of early diagnosis of Parkinson's disease in the preclinical or at least premotor phase will increase. For this reason, various studies are carried out for the development of non-invasive diagnostic methods and markers with high specificity and sensitivity. Recently, promising results have been achieved in ligand uptake-based neuroimaging techniques that demonstrate the integrity of the nigrostriatal pathway, and in marker studies performed in cerebrospinal fluid, blood, and salivary fluids [5]. Postural instability can be seen as a symptom in Parkinson's disease. This condition may indicate the deterioration of the vestibular system [6]. VEMP is one of the non-invasive and easily applicable electrophysiological tests that evaluate the inferior-vestibular nerve, brainstem and central connections starting from the saccule and macula [6]. There are studies involving the use of VEMP in diseases particularly affecting the brainstem such as multiple sclerosis, migraine, progressive supranuclear palsy, and olivopontocerebellar atrophy [7-9]. However, few studies have been conducted about VEMP in the context of evaluating the presence of brainstem pathology in IPD [7, 10, 11]. In the later stages of Parkinson's disease, loss of balance and falls can be seen due to brainstem involvement [12]. Therefore, we aimed to investigate whether vestibular functions are impaired in early and late-stage Parkinson patients through VEMP testing as neurophysiological.

2. Material and Methods

This study was carried out in the Neurophysiology Laboratory of the Neurology Department at Inonu University between January 2013 and March 2013. Before beginning the study, an ethical approval was obtained from the Local Ethics Committee of İnönü University Faculty of Medicine. In this study, 55 patients followed up with the diagnosis of IPD in the Movement Disorders Outpatient Clinic of the Department of Neurology of Inonu University, and 24 age- and gender-matched healthy volunteers as the control group were enrolled. The IPD patients were grouped as early stage and late stage according to Hoehn-Yahr staging. All patients and healthy volunteers were informed about the method and purpose of the study, and each participant signed an informed consent form. The patients with IPD were questioned in terms of brainstem symptoms, and it was purposed to assess whether the VEMP test could provide a diagnostic contribution especially in the early stage.

The patients and healthy volunteers were examined via otoscope before the VEMP examination especially in terms of neck movements. The patients with abnormal otoscopic examination or problems in neck movements, those with hearing threshold above 20 dB and conductive hearing loss in the audiometric test were not included in the study.

The room in which the examination was performed was well ventilated, dimly lit, and kept at a constant temperature of 25 °C. During the recording, the subjects were asked to be relaxed but awake, and to look at a fixed point with their eyes open. A click sound was given to the ear with the help of auditory stimulus to evoke VEMP. The inclusion criteria for the study were determined separately for each group. In the IPD group, the patients with definite diagnosis of idiopathic Parkinson's disease according to the diagnostic criteria established by Hughes et al. and those without clinical or electrophysiological peripheral neuropathy that could affect VEMP testing were included in the study. In the control group, the subjects who did not have any complaints and had normal neurological examination were enrolled in the study.

2.1. *VEMP Protocol*

The VEMP examination was performed with the Medtronic EMG-EP device (version 4.3.505.0-Model 190B6). The examination was performed while the participant was asked to turn her/his head to the opposite side of the stimulated ear and always hold it in that position. For the combined muscle activation potential (CMAP) recording in VEMP examination, the active surface electrode was placed on the upper 1/3 of the sternocleidomastoid (SCM) muscle, the reference electrode was placed on the sternum, and the ground electrode was placed on the forehead region. The recording electrode impedances were kept below 5 ohms. The filter settings were adjusted to 10 Hz-3 KHz. A sound stimulus was given to each ear with headphones to evoke VEMP. The stimulus was a high intensity (105 dB HL) rarefaction click with a duration of 0.1 ms and a frequency of 3 s^{-1}. The procedure was

performed at least twice to ensure reproducibility in each ear and 128 CMAPs were averaged. Since high-intensity sound was used, a special attention was paid to the placement of the headphones during the recording.

The presence of a positive wave with a latency of approximately 13 ms (P13) and a negative wave with a latency of approximately 23 ms (N23) were investigated as the first reflex response in the VEMP examination. The latencies of these potentials were determined with the marker. The absolute amplitude of the P13-N23 component was measured.

2.2. Statistical Analysis

The "IBM SPSS Statistics Ver. 20 for MAC" statistical software package was used for statistical analyses of the data. In the statistical evaluation, the chi-square test for categorical variables, Student's t-test for continuous variables, and multiple logistic regression analysis for correlation analysis were performed.

3. Results

The ages of the patients ranged from 47 to 84 (Mean±SD: 66.95±8.83) and showed no statistical difference with the control group. Thirty-four of the patients were male, and 21 were female, and the gender distribution between the patients and healthy controls was similar (Table 1). Of the patients with IPD, 26 were in early stage and 29 was in late stage according to the Hoehn-Yahr staging.

Table 1: Age, gender and VEMP values of the Parkinson patients and control group.

	Control n=24	IPD patients n=55	p-value
Age	63.38±8.90	66.95±8.83	0.108*
Gender F/M	11/13	21/34	0.347**
Right P13 latency (ms)	13.48±2.03	14.14±2.58	0.227*
Right N23 latency (ms)	18.45±3.03	19.11±3.46	0.392*
Left P13 latency (ms)	14.55±2.74	14.39±2.44	0.812*
Left N23 latency (ms)	19.12±3.27	19.43±3.20	0.702*
Right P13-N23 amp (μV)	7.52±5.43	3.63±3.04	0.003*
Left P13-N23 amp (μV)	7.35±6.45	4.99±4.33	0.110*

*t test, ** chi-square test.

There was no significant difference between the Parkinson patients and the control group in terms of the mean values of right and left VEMP P13 wave latency (p=0.227, p=0.812, respectively) and N23 wave latency (p=0.392, p=0.702, respectively). The right and left P13-N23 amplitudes of the Parkinson patient group were lower than those of the control group. However, this difference was statistically significant only for the right P13-N23 amplitude.

The Parkinson patients were evaluated by grouping them according to the presence of falls. It was determined that falls were present in 29 patients (Table 2). Among the patients with IPD, the P13 latency was statistically higher in those with a fall than in those without a fall (13.37±2.36, 14.83±2.61, p=0.034).

Table 2: VEMP Values According to the Presence of Falling Symptom in the Parkinson Patients.

	No fall n=26	Fall n=29	p-value*
Age (years)	64.23±8.13	69.38±8.86	0.029
Right P13 latency (ms)	13.37±2.36	14.83±2.61	0.034
Right N23 latency (ms)	18.90±3.62	19.31±3.37	0.668
Left P13 latency (ms)	14.37±2.14	14.42±2.72	0.942
Left N23 latency (ms)	19.26±2.96	19.58±3.44	0.718
Right P13-N23 amp (μV)	3.25±2.70	3.97±3.34	0.383
Left P13-N23 amp (μV)	5.31±4.56	4.70±4.17	0.610
Disease duration (month)	49.00±47.15	70.48±43.92	0.087

* t test.

The late-stage patient group was statistically older than the early-stage patient group and control group (ANOVA test p=0.025, post hoc LSD test p=0.030 and p=0.014, respectively). The right VEMP P13 mean latency value in the late-stage patient group was statistically significantly prolonged than in the early-stage patient group and control group (ANOVA test p=0.043, post hoc LSD test p=0.025 and p=0.042, respectively). The mean amplitude value of the right VEMP P13-N23 in both the late stage and early-stage patient groups was statistically significantly lower than in the control group (ANOVA test p=0.000, post hoc LSD test p=0.002 and p=0.000, respectively). There was no statistically significant difference between the early-stage patient group, late-stage patient group and control group in terms of other VEMP parameters.

There was no statistically significant difference between the patient groups divided according to the presence of fall symptoms in terms of VEMP parameters and age (Table 3).

The Parkinson patients were grouped according to the presence of dementia. Dementia was present in six patients. A statistically significant difference was found between these patient groups in terms of VEMP parameters and age (Table 4).

Table 3: Age, gender and VEMP values of early and late-stage Parkinson patient groups and control group.

	Control n = 24	Early stage n = 26	Late Stage n =29	p-value*
Age (year)	63.38±8.90	64.23±8.13	69.38±8.86	0.025
Right P13 latency (ms)	13.48±2.03	13.37±2.36	14.83±2.61	0.043
Right N23 latency (ms)	18.45±3.03	18.90±3.62	19.31±3.37	0.651
Left P13 latency (ms)	14.55±2.74	14.37±2.14	14.42±2.72	0.967
Left N23 latency (ms)	19.12±3.27	19.26±2.96	19.58±3.44	0.870
Right P13-N23 amp (μV)	7.52±5.43	3.25±2.70	3.97±3.34	0.000
Left P13-N23 amp (μV)	7.35±6.45	5.31±4.56	4.70±4.17	0.156

*Anova test.

Table 4: VEMP values according to the presence of dementia in the Parkinson patients.

	No dementia group n = 49	Dementia group n = 6	p-value*
Age (year)	66.10±8.64	73.83±7.83	0.042
Right P13 latency (ms)	13.81±2.32	16.90±3.14	0.005
Right N23 latency (ms)	18.69±3.18	22.57±4.08	0.008
Left P13 latency (ms)	14.24±2.33	15.62±3.20	0.196
Left N23 latency (ms)	19.20±3.12	21.25±3.54	0.141
Right P13-N23 amp. (μV)	3.60±2.92	3.87±4.29	0.840
Left P13-N23 amp. (μV)	4.68±3.93	7.46±6.76	0.139
Disease duration (month)	57.55±45.46	83.00±51.53	0.207

* t test.

4. Discussion

Postural instability is one of the characteristic features of IPD. Injuries may occur due to falls and even disability may develop. Postural instability depends on inappropriate interaction between visual, proprioceptive, and vestibular signals [13]. In the vestibular system, the semicircular canals, utricle, and saccule in the inner ear pertain to balance. Semicircular canals assist in detecting angular motion. While the channels ensure the kinetic balance of the body, the utricle and the saccule help in perceiving linear motion and provide static balance. The utricle responds to gravity and to linear acceleration, especially in the horizontal plane. In addition, the saccule responds to linear acceleration in the foreground/background with vibrational type stimuli. Its contribution to stability and vestibular function integrity has been addressed in many studies. These studies can be listed as caloric test and rotational chair test, tilt-table, and galvanic stimulation of the vestibular system [7].

The VEMP test is an indirect test that evaluates the operation of a neuronal pathway that begins from the saccule and terminates in the SCM muscle. In other words, it neuroanatomically detects major brainstem circuits. It is thought that it examines mostly the saccular part of the vestibulospinal pathway. De Natale et al. suggested that VEMP could be a reliable method for evaluating the brainstem in Parkinson patients [14]. Scarpa et al. reported the presence of hearing loss and cervical VEMP abnormalities in high-frequency tests in both Parkinson patients and patients with multisystem atrophy, even if the patients do not have auditory and vestibular complaints [15].

It has been shown that brainstem involvement occurs in the preclinical period (Phase 1-2) when non-motor symptoms occur before the emergence of cardinal clinical manifestations of Parkinson's disease. Hence, we considered that the VEMP test might also be abnormal in early-stage Parkinson patients compared to healthy controls, and we investigated this situation in these patients. In our study, we found that the VEMP latencies in the early-stage Parkinson patients were like those of the control group, whereas there were small differences in the VEMP amplitude. However, we detected statistically significant VEMP abnormalities in the late-stage Parkinson patients than in both the control group and the early-stage Parkinson patients. Consequently, our findings indicate that VEMP impairment is more pronounced after the progression of the pathology in the brainstem in IPD.

In the study conducted with 54 IPD patients and 53 healthy controls, Pollak et al. investigated the correlation of VEMP with age, gender, disease characteristics (associated factors such as duration and stage of the disease, as well as the presence of dementia, depression, motor fluctuation, falls, dyskinesia, and psychosis) and treatment modalities. It was reported that in the IPD group, the unilateral VEMP response in 37% and bilateral response in 7.4% of the patients were not obtained. Among the patients whom the VEMP response was not obtained, 15 out of 24 patients had depression. Fifty percent of the patients without bilateral VEMP response received antidepressant treatment. It was found that there was a correlation between VEMP abnormalities and patients with depression and therefore receiving antidepressant treatment; nevertheless, there was no correlation between VEMP results and other clinical parameters [7].

In this study, there was no significant difference between the IPD group and the control group in terms

of the right and left side P13 latency and N23 latency. Although the left P13-N23 amplitude was smaller in the Parkinson patients than in the healthy controls, this difference was not statistically significant. The right P13-N23 amplitude was statistically significantly decreased in the Parkinson patients compared to the control group. Again, the right P13-N23 amplitude was statistically significantly smaller in the early-stage Parkinson patient group compared to the late-stage Parkinson patient group. It was detected that the right P13 latency was statistically significantly prolonged in the late-stage Parkinson patient group compared to the early-stage Parkinson patient group and control group.

In a study in which VEMPs in Parkinson patients and patients with progressive supranuclear palsy (PSP) were compared, a higher number of VEMP abnormalities were detected in patients with PSP, and it was suggested that the central vestibular pathways were more severely damaged in PSP than in Parkinson's disease [10]. In another study, Venhovens et al. investigated the value of VEMP as a neurovestibular test in predicting falls that may occur in the future among patients with Parkinson's disease and atypical parkinsonism. In these patients, positive predictive value of fall probability was obtained as 68% in the case of unilateral abnormal VEMP test and 83 % in the case of bilateral abnormal VEMP test [11]. In our study, similar to these previous studies, VEMP abnormalities were detected in the IPD patients with fall history.

Ampar et al. examined brainstem auditory evoked potentials and VEMP to investigate brainstem function in Parkinson patients. They found brainstem auditory evoked potentials and VEMP abnormalities in Parkinson patients, similar to our findings. They also reported that cervical VEMP abnormalities were correlated with symptoms of brainstem degeneration such as postural instability [16]. Hawkins et al. determined that advanced age, impaired proprioception, abnormal head impulse test and abnormal cervical VEMP results were correlated with deterioration in balance performance in Parkinson patients [17]. We similarly detected that there were pronounced VEMP abnormalities in the advanced stage of the disease. In a study investigating the static and dynamic otolith function and the absence of VEMP response in Parkinson patients, the absence of bilateral cervical VEMP has been found to be associated with a history of falling attacks [18]. Likewise, in another study, REM sleep behavior disorder and postural instability have been found to be correlated with VEMP abnormalities in Parkinson patients [19].

Shalash et al. studied cervical VEMP in 15 Parkinson patients and detected that cervical VEMP abnormalities of patients were significantly different than those of the control group. They stated that there were vestibular and auditory abnormalities in Parkinson patients and revealed the relationship between motor and non-motor features of the disease and brainstem dysfunction. In addition, they reported that vestibular potential abnormalities were associated with the severity and stage of the disease and recommended further studies to be conducted since VEMP may be a potential marker, especially in early-stage IPD [20]. In this study, we detected that the right VEMP P13 latency mean value in the late-stage patient group was statistically significantly prolonged than in the early-stage patient group and the control group. The right VEMP P13-N23 amplitude mean value in both the late stage and early-stage patient groups was statistically significantly smaller than in the control group. These findings indicate that the impairment in VEMP results is more pronounced in late-stage Parkinson patients, and that vestibular functions are impaired especially in advanced-stage Parkinson patients. In this study, we found that the VEMP latencies of the early-stage Parkinson patients were similar to those of the healthy controls, but there were some minor VEMP amplitude abnormalities. However, the clinical significance of these abnormalities is very limited, and thus vestibular function impairment increases in the late stage compared to the early stage of the disease.

5. Conclusions

Pronounced VEMP abnormalities were detected in the late-stage Parkinson patients. These findings show that the inferior-vestibular nerve, brainstem, and central pathway, starting from the saccule, are affected over time, especially in late-stage Parkinson patients.

Limitations of the Study

The limitations of the study is small sample size.

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Conflict of Interests

The authors declare no conflict of interest.

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Author Contributions

Conceived and designed the experiments; B.V., C.A., S.Y., K.A, O.Ç. Analyzed and interpreted the data; B.V., K.A, O.Ç. Contributed reagents, materials, analysis tools or data; B.V., C.A, S.Y., K.A, O.Ç. Wrote the paper; B.V., C.A, S.Y.

Ethical Approval

Ethics committee approval no. 2013/28 was received for this study from the ethics committee of Malatya İnönü University Faculty of Medicine Dean's Office.

Data sharing statement

All data relevant to the study are included in the article.

Consent to participate

Consent for the study was obtained from all participants for the study.

Informed Statement

The patient and control group who agreed to participate in the study signed the informed consent form.

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Determination of Antibacterial Activities of St. John's Wort (*Hypericum perforatum L.*) Oil, *Nigella Sativa* Oil, Clove (*Eugenia caryophyllata*) oil, Orange Peel (*Citrus sinensis*) and Garlic (*Allium sativa*) Oil Against Microorganisms Isolated from Clinical Samples

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Abstract: The aim of this study is to detect St. John's Wort, *Nigella sativa*, Clove, Orange Peel and Garlic oil on bacteria isolated from blood culture to determine its antibacterial effect. One hundred blood samples were sent to Atatürk University Medical Microbiology Laboratory between 1 December 2021 and 1 January 2022 and analyzed with a blood culture system. Bacteria isolated from blood culture were passaged into blood agar. The bacterial suspension was prepared from the bacterial colonies at 0.5 Mc Farland turbidity. To determine the antibacterial activity of plant extract oils, Minimum Inhibition Concentration and Minimal Bactericidal Concentration values were determined by the liquid microdilution method. Also, the zone diameters of the disc diffusion method were measured. The antibacterial effect of plant extract oils were detected on only 10 of the 100 clinical samples included in the study. St. John's Wort oil used in these ten samples showed the most effective antibacterial effect of 7.81 µg/mL against *Staphylococcus haemolyticus* and *Enterobacter aerogenes*. Garlic oil showed the most effective antibacterial effect against *Escherichia coli* and *Staphylococcus haemolyticus* at 7.81 µg/mL. *Nigella sativa* oil showed the most effective antibacterial effect against *Staphylococcus haemolyticus* at 3.9 µg/mL. Orange Peel oil showed the most effective antibacterial effect against Enterococcus faecalis at 1.95 µg/mL. Garlic oil on *Escherichia coli*, *Staphylococcus haemolyticus* and *Enterobacter aerogenes*, St. John's wort oil on *Staphylococcus haemolyticus* and Enterobacter aerogenes, *Nigella sativa* oil on *Staphylococcus haemolyticus* has been found to be effective. © 2023 NTMS.

Keywords: **Keywords:** Antibacterial Activity; Plant Extract; Blood Culture; Microdilution; Zone Diameter.

1. Introduction

Nosocomial bloodstream infections are a serious and increasing problem that causes complications parallel to bacteremia and fungemia. These infections show high morbidity and mortality due to the delay in

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managing the infectious agent with hospitalization [1-3]. In addition, the long-term hospitalization of the patient and this hospitalization increase the hospital costs. In this respect, appropriate diagnosis and treatment are very important. The determination of the treatment for the causative agent, the choice of antibiotic in the treatment and the limitation of its negative effects on the host are provided by blood culture results [4]. Blood culture is the best approach in routine laboratories to identify microorganisms and determine antimicrobial therapy when suspected of bloodstream infections [5]. After blood culture results, preliminary treatments are determined based on clinical and epidemiological data, but the response to treatment in drug-resistant organisms is uncertain [6, 7]. At this point, researchers are working on alternative treatment methods. The antibacterial activity on the studied substance/component and microorganisms has been demonstrated in vivo and in vitro. In this context, hyperforin is the active ingredient and responsible for the antibacterial effect of St. John's Wort, which has been studied extensively on microorganisms. It has been determined that the active ingredient hypericin also acts together with antibacterial activity. *Nigella sativa* is an antioxidant and has many activities. It is used as a therapeutic in infectious diseases, and it is reported that this positive effect is due to the radical-scavenging effect of essential oil [8]. Seeds of *Nigella sativa* have been used in traditional medicine for their antioxidant properties, and the oil and its active ingredients seem to reduce toxicity mediated by oxidative stress triggered by environmental or infection-related factors or anti-cancer drugs [9]. Piras et al. (2013) observed the best antimicrobial activity in *Nigella sativa* [10]. Orange peel (*Citrus sinensis*) oil contains 20.2 % linalool, 18.0 % decanol, 14.1 % citral, 5.8 % terpineol, 5.2 % valene, 4.1 % dodecanol, 3.9 % citronellol and 0.3 % limonene [11]. Limonene is one of the important active ingredients in the oil. It is available in various studies showing antimicrobial and antiseptic activity [12]. Clove (*Eugenia caryophyllata*) oil shows many pharmacological activities such as, antimicrobial and anti-cancer, based on its bioactive components such as eugenol, eugenol acetate, α-humulene, 2-heptanone, and β-caryophyllene [13]. The antimicrobial effects of garlic (*Allium sativa*) extracts are well known, however the effects of *Allium sativa* oil are little known. Many studies have been carried out to determine the antimicrobial activity of *Allium sativa*. Antimicrobial activity is greater in media without tryptone or cysteine, suggesting that, as for allicin, the effects of *Allium sativa* oil may include sulfhydryl reactivity. All tested bacteria are susceptible to garlic ingredients, including gram-negative and positive bacteria and pathogenic forms [14]. In the light of this information, we investigated the antibacterial activity of St. John's Wort (*Hypericum perforatum L.*) oil, *Nigella sativa* oil, Clove (*Eugenia* caryophyllata) oil, Orange Peel (*Citrus sinensis*), and Garlic (*Allium sativa*) oil against microorganisms isolated from blood culture samples. We aim to contribute to the treatment of nosocomial bloodstream infections.

2. Material and Methods

2.1. Clinical Isolates

One hundred blood samples sent to Atatürk University Medical Microbiology Laboratory between 1 December 2021 and 1 January 2022 were analyzed with the BacTAlert (Biomerieux, France) blood culture system. Identification and antibiotic susceptibility of the growing isolates were studied with automated Vitek version 2.0 (Biomerieux, France). One hundred bacterial species identified in line with the cultivation of selective agars from the bottles were included in the study. Control of these bacteria was done with standard strains. Bacteria isolated from blood culture were passed. In accordance with Clinical Laboratory Standards Institute (CLSI) recommendations, a bacterial suspension was prepared from 24-hour bacterial colonies, equal to 0.5 Mc Farland turbidity [15, 16].

2.2. Preparation of the Medium

Microorganisms were inoculated on Eozyne Methylene Blue and blood agar to obtain new cultures from the stock culture to be used in antibacterial activity tests. A commercially purchased Tryptic Soy Broth medium to be used to determine the Minimum Inhibition Concentration (MIC) was prepared at 21 g/l in distilled water.

2.3. Preparation of Plant Extract Oils

In studies on surfactants, plant extract oils dissolve easily. Substances such as Tween 20, Tween 80 and Sodium dodecyl sulfate (SDS) have positively affected antibacterial activity [17-19]. Kuang et al. (2018) In their study, it was determined that the essential oil dissolved in Tween 20 has the strongest antibacterial activity [17]. In line with the studies, Tween 20 was preferred as the solvent. In this study, plant extract oils for antibacterial tests were prepared by filtering through 0.22 μm filters with a solution concentration of 10 % (v/v)[17].

2.4. Antibacterial Activity Tests

2.4.1. Determination of the Minimum Inhibition Concentration (MIC)

CLSI was applied for the identification and valuation of MIC [16]. St. John's Wort oil, *Nigella sativa* oil, Clove oil, Orange Peel, and Garlic oil were dissolved with Tween 20 to determine the final concentration of 500 μg/ml. In the Minimum Inhibition Concentration Determination method, ten microorganisms taken from Eosin Methylene Blue and blood agar were taken to the study and 24-hour fresh culture. The colonies taken from the prepared cultures prepared a bacterial suspension in sterile 0.9 % saline with a turbidity of 0.5 Mc Farland $5x10^5$ CFU/ml. Then, 100 μl of Tryptic Soy Broth medium was added to all wells 1 to 12 of the sterile 96-well microplate. First, 100 μl of the relevant commercial plant oil (500-50 μg/ml) was added to the

1st well, and 100 µl dilution was made up to the 10th well in a 1:1 ratio. Then, 100 µl of bacterial suspension was added to the 10th well. Bacteria control only bacterial suspension was added to the well, and only commercial plant extract oils were added to the 12th well. Then, the microplate was covered with parafilm and incubated for 24 hours at 37 °C. The last well without growth was determined as the Minimum Inhibition Concentration (MIC) value. The antibacterial effect of plant extracts was tested by minimum inhibitor concentrations (MIC) against ten control bacterial strains Gentamicin was used to control the study [20-24].

2.4.2. Determination of Minimal Bactericidal Concentration (MBC)

The lowest antibiotic concentration without turbidity was accepted as the MIC value. To determine the MBC values, ten µl was taken twice from all wells that did not show growth at the end of the incubation period. Colonies formed as a result of incubation were counted, and the lowest antibiotic concentration that killed 99.9 % of the initial inoculum was determined as the MBC value.

2.4.3. Kirby Bauer Disc Diffusion Method

Microorganism colonies taken from 24-hour cultures were adjusted in sterile saline to Mc Farland 0.5 turbidity and inoculated into Mueller Hinton medium with a swab stick. Plant extract oils were dissolved in Tween 20. Then, 6 mm diameter Sterile Paper Discs (Oxoid, Oxoid Antibacterial Susceptibility Blank Test Disc, Hampshire, UK) were placed on a Muller Hinton Medium containing an inoculated culture medium, then a 20 µg/mL sample was impregnated into the Oxford plate; 10 % Tween 20 solvent was used as a blank control. Gentamicin (10 µg/mL) was used as a positive. Zone diameters were measured after 24 hours of incubation [20-24].

3. Results

The antibiotic susceptibility results obtained from the automated system VITEK 2 (bioMerieux/ France) device were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [25].

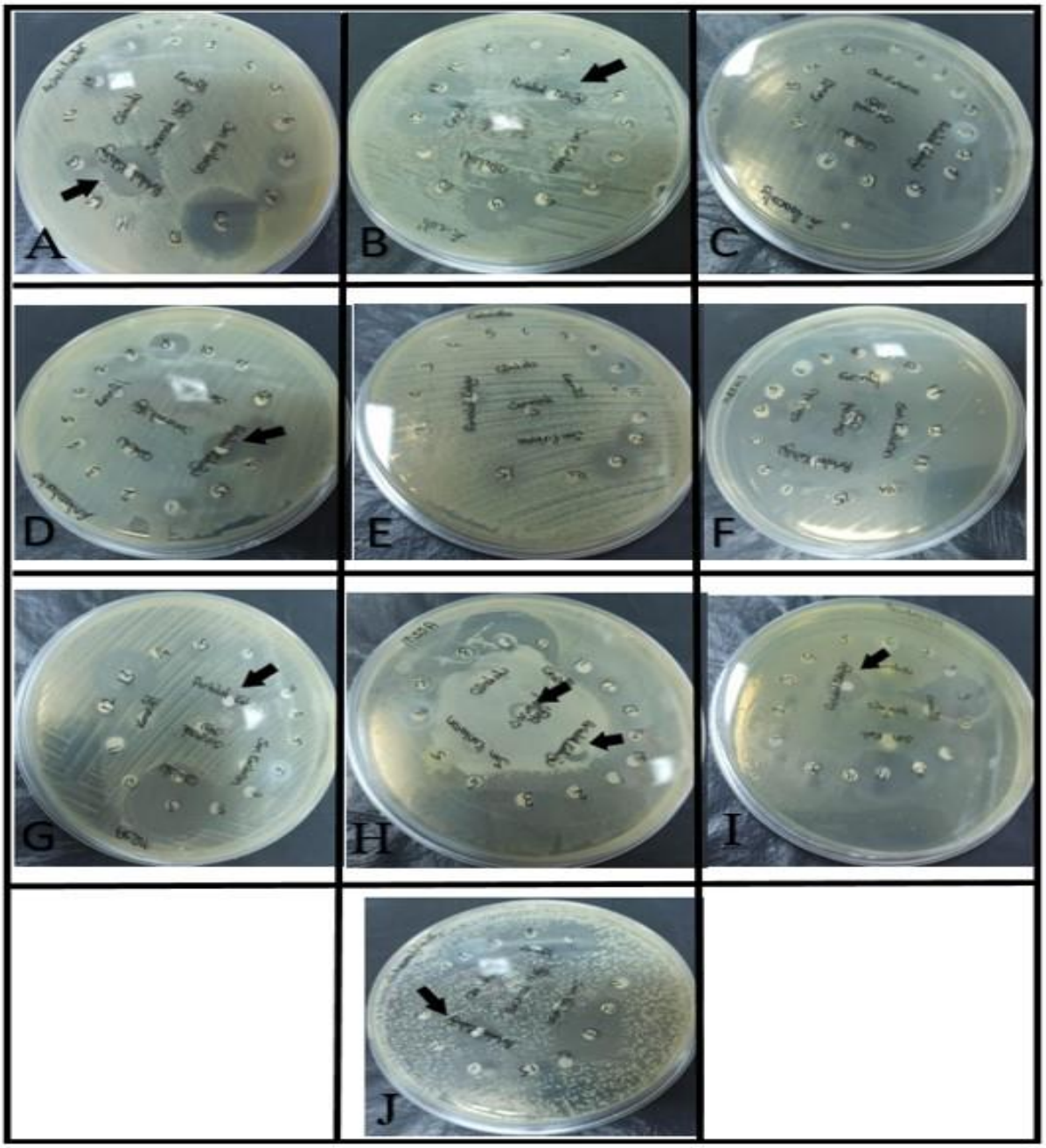
In our study, antimicrobial susceptibility testing of bacteria isolated from blood cultures using plant extract oil was performed by liquid microdilution method. The antibacterial effect of plant extract oils was detected on only 10 of the 100 clinical samples included in the study. No antibacterial activity was detected on the other 90 samples. The antibacterial effect of 10 microorganisms was determined, and MIC and MBC values are shown in Table 1. The lowest antibiotic concentration without turbidity was accepted as the MIC value. Colonies formed as a result of incubation were counted, and the lowest antibiotic concentration that killed 99.9 % of the initial inoculum was determined as the MBC value. The effect of plant extract oils on standard bacterial strains used in the study was evaluated by MIC, MBC and Kirby Bauer Disc Diffusion method. Kirby Bauer Disc Diffusion method determined all bacterial strains resistant to plant extract oils. In addition, the MIC value was determined as 31.25 µg/mL only in *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC700603 strains.

In our study, Kirby Bauer disc diffusion zone diameters of 10 microorganisms showing antibacterial activity are shown in Figure 1, and their sensitivity to oils is shown in Table 2.

4. Discussion

In the present study, antibacterial activity of plant extract oils (St. John's Wort (*Hypericum perforatum L.*) oil, Nigella sativa (*Nigella sativa*) oil, Clove (*Eugenia caryophyllata*) oil, Orange Peel and Garlic (*Allium sativa*) oil were investigated by determining the MIC, MBC values and inhibition zones on agar plates. All the tests determined that Orange Peel (Citrus sinensis) had inhibitory properties.

Orange Peel oil could not show antibacterial activity against *Klebsiella pneumoniae*, MRSA, and *Staphylococcus haemolyticus.* Orange Peel (*Citrus sinensis*) oil showed the most effective antibacterial effect against *Enterococcus faecalis* at 1.95 µg/mL. In a study on the in vitro antibacterial effect of Orange Peel essential oil against bacterial fish pathogens, the effect of the oil on microorganisms was examined, respectively, *L. anguillarum, Y. ruckeri, V. alginolyticus, V. salmoninarum, A. hydrophilave L. garvieae* has also been determined. It was determined that the pathogen in which the obtained essential oil showed the strongest antibacterial activity was *L. anguillarum* at 10% concentration. When the microdilution results were evaluated, the highest MIC value was found in *L. anguillarum* (31.25 µg/ml), followed by *Y. Ruckerive, V. salmoninarum* (62.5 µg/ml), *A. hydrophilave, V. alginolyticus* (125 µL/mL) respectively, followed by *L. garvieae* (250 µg/ml). It has been determined that orange oil is effective against *L. anguillarum*, which is the most effective bacteria, at a dose of 31.25 µg/ml [26].



*A. *Acinetobacter spp. Citrus sinensis* oil (25 mm) zone diameter, B. *Escherichia coli Citrus sinensis* oil (20 mm) zone diameter, D. *Enterobacter aerogenes Citrus sinensis* oil (14 mm) zone diameter, G. MRSA *Citrus sinensis* oil (10mm) zone diameter, H. MSSA *Citrus sinensis* oil (10 mm) and *Allium sativa* oil (10 mm) zone diameter, I. *Pseudomonas aureginosa Citrus sinensis* oil (12 mm) zone diameter, J. *Staphylococcus heamolyticus Citrus sinensis* oil (16 mm) zone diameter

Figure 1: Antibacterial zone diameters of plant extract oils by disk diffusion method.

Table 1: Minimum Inhibition Concentration and Minimal Bactericidal Concentration Values.

Clinical Isolates /*Plant Extracts*	**St. John's Wort oil**		**Nigella sativa oil**		**Clove oil**		**Orange Peel oil**		**Garlic Oil**	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
E. coli	125	93.75	62.5	46.87	125	93.75	125	93.75	7.81	5.855
K. pneumoniae	1.95	1,46	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	125	93.75
P. aeruginosa	31.25	23.42	62.5	46.87	62.5	46.87	62.5	46.87	62.5	46.87
E. faecalis	125	93.75	500 ≤	500 ≤	250	187.5	1.95	1.46	15.62	11.71
MSSA	250	187.5	250	187.5	250	187.5	125	93.75	125	93.75
MRSA	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	500 ≤	250 ≤	500 ≤
MRCNS	500 ≤	500 ≤	500 ≤	500 ≤	125	93.75	250	187.5	31.25	23.42
S. haemolyticus	7.81	5.85	3.9	2.44	500 ≤	500 ≤	500 ≤	500 ≤	7.81	5.855
E. aerogenes	7.81	5.85	500 ≤	500 ≤	250	187.5	250	187,5	3,9	2.44
A. baumannii	125	93.75	125	93.75	125	93.75	62.5	46.87	500 ≤	500 ≤

*Ten clinical samples were included in the study, St. John's Wort oil used in our study showed the most effective antibacterial effect of 7.81 µg/mL against *Staphylococcus haemolyticus* and *Enterobacter aerogenes*. *Nigella sativa* oil showed the most effective antibacterial effect against *Staphylococcus haemolyticus* at 3.9 µg/mL. *Orange Peel* oil showed the most effective antibacterial effect against *Enterococcus faecalis* at 1.95 µg/mL.

Table 2: Plant Extract Oil Sensitivity.

	P. aeruginosa			***K. pneumoniae***			***E. faecalis***			**MSSA**			**MRSA**			***E. coli***			**MRCNS**			**S. *haemolyticus***			***E. aerogenes***			***A. baumanii***		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
1			x			x			x			x			x			x			x			x			x			x
2			x			x			x			x			x			x			x			x			x			x
3			x			x			x			x			x			x			x			x			x			x
4			x			x			x			x			x			x			x			x			x			x
5	x					x			x			x		x			x			x		x			x			x		

1- St. John's Wort (*Hyperic um perforatum L.*) oil, 2- *Nigella sativa* oil, 3- Clove (*Eugenia caryophyllata*) oil, 4- Garlic (*Allium Sativa*) Oil, 5- Orange Peel (*Citrus sinensis*) oil.

*S: Sensitive, I: Intermediate, R: Resistance.

In our study, clove oil showed a MIC value of 62.5 µg/mL as effective on *Pseudomonas aureginosa*. It did not show any inhibitory properties on any microorganisms in the Kirby Bauer Disc diffusion method.

Emeka et al. (2015) examined black cumin oil against *Staphylococcus aureus* isolated from the wounds of 34 diabetic patients at varying concentrations by pit diffusion method. While 8 of 19 isolates were sensitive to undiluted oil samples, 4 were sensitive to 200 mg/mL, 400 mg/mL, and 800 mg/mL, and 11 were resistant to all oil concentrations. They found that more than half of the isolates were sensitive to black cumin oil at different concentrations [28]. Disc diffusion method, which we used to determine the antibacterial activity of black cumin oil against ten microorganisms, we were unable to detect any inhibitory effect against any microorganisms. We believe this is not the effective amount of the stock solution (500 µg/ml) we used compared to the studies.

Allium sativum L. (Kastamonu and Denizli local) in a study on the comparison of the chemical compound, the antibacterial and antioxidant activity of essential oils of the plant, the inhibition zone of *Escherichia coli* MC 4100 strain was 13 and 12 mm, *Pseudomonas aeruginosa*, in which Garlic essential oil was applied as 30 µl/disc. *Pseudomonas aeruginosa* NRRL-B-2679 strain, 6 and 5 mm, *Enterobacter aerogenes* NRRL-B-3567 strain 9 and 7 mm, *Staphylococcus aureus* ATCC 33862 strain 9 and 11 mm; In the petri dish with 50 µl/disk, the inhibition zone was measured as 15 and 15 mm. *Escherichia coli* MC 4100 strain 15 and 15 mm, Pseudomonas aeruginosa in a petri dish containing 50 µl disc. *Pseudomonas aeruginosa* NRRL-B-2679 strain was 11 and 10 mm, *Enterobacter aerogenes* NRRL-B-3567 strain was 15 and 14 mm, *Staphylococcus aureus* ATCC 33862 strain was 12 and 14 mm [29]. Kim et al. (2004) while they found the antimicrobial activity of the garlic oil obtained to be high, and O'Gara et al. (2000) found that the isolated was low [30, 31]. In our study, while 10 mm zone diameter was detected only on MSSA in the disc diffusion method, it could not show any antibacterial activity on other microorganisms.

In the present study, no inhibition zone was observed on agar plates of Tween 20 extract of *Hypericum perforatum L.* against microorganisms; *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, MSSA, MRSA, *Escherichia coli*, MRCNS, *Staphylococcus haemolyticus*, *Enterobacter aerogenes* and *Acinetobacter baumannii*. In this context, the two studies show parallels with each other. The fact that another does not support only the use of the disc diffusion method in vitro study is incomplete in evaluating the results. However, in our study, observing antibacterial activity against many microorganisms can be based on the fact that the strain used is not a reference strain and therefore can be resistant to the extracts used (not tried against comparative antibiotics). Chemicals used as solvents are likely to differ in studies with essential oils. Due to the fact that plant essential oils have different chemical structures, not every oil dissolves to the same degree in the same solvent. On the other hand, since the solvent used is also different, this difference may also be associated with the solvent's ability to decode active compounds.

5. Conclusion

Garlic (*Allium sativa*) oil on *Escherichia coli*, *Staphylococcus haemolyticus* and *Enterobacter aerogenes*, St. John's Wort oil on *Staphylococcus haemolyticus* and Enterobacter aerogenes, *Nigella sativa* oil on *Staphylococcus haemolyticus* has been found to be effective. We think more studies are needed to determine the effect of other doses and time. Studies should shed light on what components might be responsible for the antimicrobial activity of these extracts against target isolates.

Limitations of the Study

The fact that no antibacterial activity was found in 90 out of 100 samples in the study leads to the thought that higher ranges should be selected from these dose ranges in future studies. In addition to Tween 20, other solvents can be preferred as a degreaser. Low dose and solvent type were limitations of the study.

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Conflict of Interests

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Author Contributions

Conceptualization, ÖÇ, SB, MCG, DÇ and S.Ç; methodology, ÖÇ, SB, MCG, DÇ and S.Ç; validation, ÖÇ, SB, MCG, DÇ and S.Ç; formal analysis, ÖÇ, SB, MCG, DÇ and S.Ç; investigation ÖÇ, SB, MCG, DÇ and S.Ç; resources, ÖÇ, SB, MCG, DÇ and S.Ç; data curation ÖÇ, SB, MCG, DÇ and S.Ç; writing-original draft preparation, ÖÇ, SB, MCG, DÇ and S.Ç; writing-review and editing, ÖÇ, SB, MCG, DÇ and S.Ç; visualization ÖÇ, SB, MCG, DÇ and S.Ç; supervision ÖÇ, SB, MCG, DÇ and S.Ç; project administration ÖÇ, SB, MCG, DÇ and S.Ç.

Ethical Approval

Ethical permission was obtained from the Atatürk University Medical Faculty Clinical Research Ethics Committee for this study with date 04.11.2021 and number 07, and Helsinki Declaration rules were followed to conduct this study.

Data sharing statement

Not applicable.

Consent to participate

Not applicable.

Informed Consent
Not applicable.

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The Effect of Long-Term Lithium Use on Renal Functions in Patients with Bipolar Disorder

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Abstract: It is known that especially glomerular side effects of lithium lead to serious consequences such as end-stage renal disease. Therefore, it is critical to evaluate patients on long-term lithium therapy for glomerular pathologies. The present study investigated the changes in renal functions, prevalence of renal failure and progress in patients have been followed up for at least six years with bipolar disorders (BD) and on regular lithium treatment. 51 patients with BD and 38 age and sex matched healthy controls were enrolled for the study. The serum blood urea nitrogen (BUN), creatinine, uric acid, electrolytes, calcium (Ca), phosphorus (P), vitamin D (25-OH D3), parathyroid hormone (PTH) and eGFR levels were measured to compare the kidney functions of patients and control group. The relation between the renal functions and mean serum lithium levels and duration of lithium treatment were also investigated. Mean eGFR level, 25-OH D3 and urine density of patients with were significantly lower whereas creatinine, uric acid, Ca and PTH were significantly higher than that of controls. The duration of lithium treatment and mean lithium levels were negatively correlated with eGFR level. Eight of 51 patients have critical eGFR level as lover than 60ml/minute thus further nephrological investigation was needed. The study revealed that the renal functions of the patient group was significantly lower than controls. The findings suggested that both duration of lithium treatment and high serum lithium levels may have a negative impact on renal functions. These findings suggest that it is important to clarify the response type to lithium in patients who are on long term treatment with lithium and maintain the treatment with the lowest possible therapeutic serum levels and carefully monitoring the renal functions in patients with good response to lithium. © 2023 NTMS.

Keywords: Lithium; Chronic Kidney Disease; Bipolar Disorder; Glomerular Filtration rate.

1. Introduction

Lithium, which has been in the treatment of bipolar disorder for more than 70 years, has been proven to be effective in the treatment of both depressive and manic episodes at the present time [1]. Lithium, which is

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known to reduce the suicide rate significantly and has superiority over other mood-stabilizing drugs in this aspect, is also accepted as the first choice in the maintenance treatment of bipolar disorder (BD) [2].

Despite its effectiveness in the treatment, the side effects of lithium on the thyroid gland and kidneys are feared of. Side effects on kidney functions are divided into two groups as effects on tubular functions and effects on glomerular functions. Lithium is the most common cause of drug-induced nephrogenic diabetes insipidus (NDI), and studies on this subject report an incidence between 20 % and 87 % [3]. The first publications on whether lithium causes chronic renal failure (CRF) are based on case reports [4]. As a result of kidney biopsies performed on patients using lithium, drug-induced chronic tubulointerstitial nephritis (CTIN) was defined. In a study published in 2000, in all 24 patients who received lithium treatment for an average of 13.6 years and underwent renal biopsy due to renal failure, CTIN characterized by tubular atrophy and interstitial fibrosis was detected; It has been reported that end-stage renal failure (ESRD) developed in 8 of 19 patients followed up despite stopping lithium treatment, and initial serum creatinine levels were important in the development of ESRD [5]. Tubular cyst formation detected by imaging methods is thought to be an indicator of lithium-induced nephropathy [6].

The incidence of lithium-induced ESRD is estimated to be as low as 0.2% to 0.7% [7]. However, these rates are approximately eight times higher compared to the general population and cause controversy regarding lithium use [8].

Glomerular functions are evaluated by calculating serum creatinine level, creatinine clearance and glomerular filtration rate (GFR). Early studies reported that long-term lithium use did not cause a significant change in GFR [9]. The prevalence of decreased GFR was found to be 15 % in a meta-analysis of 14 studies published before 1987 [10]. However, in subsequent studies, between 21 % and 55 % of patients treated with lithium were found to have GFR levels <60 ml/min/1.73 m^2 (stage 3 renal failure cut-off point) [11]. A decrease in GFR was detected in 21 % of patients using lithium for at least 15 years [12], and in another study, 21 % of patients using lithium for a long time had serum creatinine levels of 1.5 mg/dl and above in two consecutive measurements, and it has been emphasized that the risk of renal failure is increased after the 15th year of treatment [13]. On the other hand, in a recent study conducted with participants matched for age, gender, and baseline GFR, it was reported that lithium and control groups did not differ in terms of reductions in GFR levels, and it was thought that methodological differences in studies reporting the opposite opinion might have affected the result [14]. Also, in a recently done meta-analysis, it was reported that the decrease in GFR levels due to lithium treatment was not clinically significant in most patients and that medical comorbidities frequently found in BD patients may be effective in the development of renal failure [15]. Despite different results, it is a dominant view in the literature and a serious clinical problem that lithium leads to the development of CRF by causing CTIN [16].

Based on the current data we have, it does not seem possible to predict ESRD that may develop in patients using lithium other than knowing some risk factors such as taking lithium treatment for a long time, advanced age, low initial GFR, history of lithium intoxication, presence of nephrogenic diabetes insipidus, presence of additional medical diseases that increase the risk of CRF such as hypertension and diabetes mellitus, use of nephrotoxic drugs. Current guidelines on monitoring and managing kidney functions in clinical follow-up are insufficient.

Although the renal tubular side effects of lithium have been clearly demonstrated, there are uncertainties regarding glomerular side effects with more serious consequences. Therefore, it seems important to determine the development of chronic renal failure and the factors that predispose it, especially in patients under long-term lithium treatment

In this study, it is aimed to identify the effects of long-term lithium treatment on kidney functions in BD patients, whether other metabolic and endocrine system parameters such as parathormone (PTH), serum calcium, 25-hydroxyvitamin D3 (25-OH D3) levels have predictive effects on lithium use and kidney functions, and associated factors.

2. Material and Methods

2.1. Sample

This cross-sectional study was conducted with 51 patients diagnosed with BD according to DSM-V diagnostic criteria, and 38 healthy volunteer participants without a psychiatric diagnosis who were matched for age and gender. In the power analysis performed with 80 % power and 0.05 alpha level based on the creatinine clearance loss per year in the reference study [17], the minimum and the maximum number of samples to be included in the study were determined as 56 and 94 respectively. Patients who were followed up in Istanbul University Faculty of Medicine, Department of Psychiatry, using lithium regularly for at least 6 years and whose retrospective information such as medical data and mood graphs were well recorded, and those who gave voluntary consent to the study were included in the study. A detailed history about internal diseases was taken from the patients and healthy controls, and the patients who used lithium irregularly, had a known kidney disease and were on dialysis as a result of lithium intoxication, and participants from the control group with known psychiatric disorders, severe internal or kidney diseases were excluded. All bipolar patients on long-term lithium therapy were included in the study, and a second analysis was performed by excluding participants with confounding factors such as DM and antihypertensive drug use that may affect glomerular functions. The protocol of the study was approved by Istanbul University Non-Interventional Clinical Research Ethics Committee (File no:

2015/1999) and written informed consent was obtained from all participants.

2.2. Data Collection Tools

The participants' sociodemographic and clinical characteristics, medications, responses to lithium, alcohol and substance use, and psychiatric histories were recorded in the semi-structured interview form.

The treatment response of the patients to lithium was determined by the mirror model method. By using the lifelong mood monitoring charts in the patients' files, the pre-lithium period and the lithium treatment period were compared and the type of response to lithium was examined in three categories. Accordingly, good response of lithium response; no mood episodes during treatment, moderate response of lithium response; when compared to the pre-lithium period, a decrease in the frequency, duration and severity of mood episodes was observed in the lithium-using period, and a poor lithium response was defined as no decrease in the frequency, duration and severity of mood episodes. In order to calculate the mean lithium blood level of the patients, the arithmetic mean of the lithium blood levels, which were checked regularly during the follow-up period, was taken.

In our study, 10 ml of venous blood and spot urine samples were taken from the patients and the control group after 10-12 hours of fasting to evaluate the kidney functions.

2.3. Evaluation of Kidney Functions

GFR is considered as the best indicator of kidney function and calculation of it is based on serum creatinine. In this study, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was used to calculate the estimated GFR (eGFR) values of the patients. The CKD-EPI formula includes serum creatinine values, patient's age, gender, and race variables [18].

2.4. Measurement of Biochemical Parameters

The following parameters were examined by respective methods. Fasting blood sugar (FBS): by the hexokinase method; HBA1C: by high performance liquid chromatography (HPLC) method; creatinine and protein in urine: by the immunoturbidimetric method; blood urea nitrogen (BUN): by kinetic UV test; serum creatinine: by the colorimetric Jaffe method; serum Sodium (Na), Potassium (K), and Chlorine (Cl): by indirect ion selective electrode (ISE) method; serum Magnesium (Mg): by colorimetric method; serum Calcium (Ca) and Phosphorus (P): by photometric method; parathormone (PTH), thyroid stimulating hormone (TSH), anti-TPO, anti-TG: by the ECLIA (Electrochemiluminescence Immunassay) method; Vitamin D (25-OH D3): by ultrahigh performance liquid chromatography (UHLPC) method.

2.5. Statistical analysis

IBM SPSS 21 Package Program was used in the statistical evaluation of the data. Data are presented as mean, standard deviation, median, minimum, maximum, percentage and number. The normal distribution of continuous variables was analyzed using the Shapiro Wilk test. In the comparisons between two groups with numerical variables, the Independent Samples T test was used when the normal distribution condition was met, and the Mann Whitney U test was used if it was not. In the comparison of continuous variables with more than two groups, the ANOVA test was used when the normal distribution condition was met, and the Kruskal Wallis test was used when it was not. The comparison between categorical variables was made with Chi-square test and Fisher's Exact test. In the comparison of two continuous variables, Pearson correlation test was used if the normal distribution condition is met, and the Spearman correlation test was used if it was not, and the statistical significance level was accepted as $p<0.05$.

3. Results

51 BD patients and 38 healthy control groups were included in the study, and both groups were matched for age and gender. There were 35 women and 16 men in the patient group; 27 female and 11 male participants in the control group, and the mean ages were calculated as 51.47±11.41 and 48.92±11.77 in the patient and control groups, respectively. (p=0.307).

The mean duration of lithium treatment of the patients was 205.41±95.83 months, and the patient who received lithium treatment for the shortest period of time used it for 72 months and the patient who received lithium treatment for the longest period of time used it for 396 months. There are 17 participants with a good response to lithium, 31 with a moderate response, and 3 participants with a poor response.

Patients whose lithium blood levels were measured above 1.2 mEq/L at least once during their follow-up were included in the patient group with a history of lithium intoxication, and a history of lithium intoxication was found in 13 (25.49 %) patients. While the treatment of 17 patients (33.33 %) was continued with lithium alone, the treatment of 34 patients (66.66 %) was continued with at least 2 psychotropic drugs. The clinical features of the patient group are shown in Table 1.

Biochemical parameters were compared between the groups and eGFR levels were found to be significantly lower in the patient group. Serum creatinine, uric acid, Ca, Cl, Mg, PTH, Anti-TG and urinary creatinine parameters were significantly higher in the patient group; 25-OH D3, HGB, HCT, and urine density were found to be significantly lower in the patient group. The comparison of the groups in terms of biochemical parameters is shown in Table 2.

Table 1: Clinical characteristics of the patient group.

Clinical Variable	Yes (N ,%)	No (N, %)	Total (N, %)
Psychotic Feature	42 (82.35 %)	9 (17.65 %)	51 (100 %)
History of Lithium Intoxication	13 (25.49 %)	38 (74.5 %)	51 (100 %)
Lithium Monotherapy	17 (33.33 %)	34 (66.66 %)	51 (100 %)
Good Response Rate to Lithium	17 (33.33 %)	34 (66.66 %)	51 (100 %)
Psychiatric Co-Diagnosis	6 (11.76 %)	45 (88.24 %)	51 (100 %)
History of Suicide Attempt	10 (19.6 %)	41 (80.4 %)	51 (100 %)
Alcohol Use	9 (17.64 %)	42 (82.36 %)	51 (100 %)
Smoking	24 (47.05 %)	27 (52.95 %)	51 (100 %)
History of ECT	22 (43.13 %)	29 (56.87 %)	51 (100 %)
Thyroid Hormone Replacement	22 (43.13 %)	29 (56.87 %)	51 (100 %)

ECT: Electroconvulsive Therapy.

While hypercalcemia (Serum Ca>10.4 mg/dl) was detected in 6 individuals in the patient group, hypercalcemia was not detected in any individual in the control group (p=0.036). BUN and Anti-TPO variables did not show a statistically significant difference between the two groups. While proteinuria was detected in 4 people, there was no participant in the control group with proteinuria, but this difference did not have statistical significance.

Correlations between age, duration of lithium use, mean lithium levels and serum PTH levels and parameters reflecting renal functions were examined. As a result of the analysis, there was a weak correlation between age and BUN and serum creatinine; a moderate and inverse correlation was found between age and eGFR. A weak correlation between the lithium-use duration and BUN, Ca, and urine density; a moderate correlation between uric acid, serum creatinine and eGFR were found. There was a weak correlation between mean lithium blood level and serum creatinine and eGFR.

A weak correlation was found between PTH levels and uric acid, serum creatinine, urine density and eGFR variables. Correlations between clinical variables and biochemical parameters and kidney functions are shown in Table 3.

The mean lithium blood levels of the patients for the last 1 year were determined, and the parameters reflecting the kidney functions of the patients whose mean lithium blood level was above and below 0.8 mEq/L were compared. The serum creatinine and PTH values of the patients whose lithium blood levels were above 0.8 mEq/L were found to be statistically significantly higher. Comparison of serum PTH and creatinine levels of patients divided into two groups according to mean lithium level is shown in Table 4.

The biochemical values of patients who responded well and moderately/poorly to lithium treatment were compared, but no significant difference was found between the groups in any parameter.

The results in Table 5 were obtained when the kidney functions were re-evaluated by excluding the participants who used oral antidiabetic and/or antihypertensive drugs and whose HBA1C values were >5.6 mg/dl.

According to the results obtained, serum creatinine, uric acid, PTH, and Ca parameters were significantly higher in patients; eGFR, 25-OH D3, urine density, and urinary creatinine parameters were found to be significantly lower.

The eGFR level of 8 out of 51 patients was found to be below 60 ml/min/1.73 m2, which is prognostically important, and these patients were referred to the nephrology outpatient clinic for examination and treatment.

4. Discussion

4.1. Findings Related to Glomerular Functions

Although the view that lithium is one of the causes of chronic renal failure is not widely accepted [10], epidemiological and clinical data indicate that lithium increases the risk of chronic renal failure [19]. However, the details about the incidence, process and severity of this clinical presentation are unclear. Serum creatinine elevations are detected in some patients, which are seen in advanced ages and can be stabilized by reducing the lithium dose; whereas other patients may experience elevations in serum creatinine levels and progressive renal failure despite discontinuation of lithium therapy [3]. In the light of the analyzes performed, the only significant indicator in the progression to ESRD was that a serum creatinine level of 2.5 mg/dl and above at the time of biopsy was considered as a factor predicting ESRD [5]. In a study, it was stated that the probability of recovery of impaired renal function is higher in patients with a creatinine clearance above 40 ml/min when lithium is discontinued, and the deterioration in renal function continues progressively in most patients with a creatinine clearance below 40 ml/min [17].

Table 2: Comparison of biochemical parameters of patient and control groups.

	Patient Group				Control Group					
	N	Mean	SD	Median	N	Mean	SD	Median	t , Z*	p
Creatinine	51	.91	.40	.80	38	.70	.16	.67	3.493	<.001
Uric Acid	46	5.8	1.5	5.9	38	4.7	1.3	4.5	3.662	<.001
eGFR	51	87.47	25.57	95.00	38	103.68	16.38	108.16	3.393	.001
Cl	50	104.06	2.82	104.00	38	102.00	2.27	102.00	3.683	<.001
Ca	51	9.88	.40	9.90	38	9.46	.33	9.50	5.246	<.001
Mg	49	.94	.36	.89	36	.83	0.05	0.84	2.277	0.023
PTH	50	79.92	72.00	69.00	38	48.46	19.75	44.08	3.782	<.001
Urine Density	50	1010	5	1008	36	1016	8	1015	3.827	<.001
Creatinine Urine	48	76.75	52.36	53.29	34	131.60	97.31	104.73	2.805	0.005

Table 3: Relationship between clinical variables and biochemical parameters and kidney functions.

		BUN	CRE	EGFR
Lithium Use Duration	Correlation Coefficient	.481	.698	-.571
	p	<0.001	<0.001	<0.001
	N	51	51	51
Age	Correlation Coefficient	.482	.375	-.617
	p	<0.001	0.007	<0.001
	N	51	51	51
Mean Lithium Level	Correlation Coefficient	.184	.344	-0.306
	p	0.184	0.013	0.029
	N	51	51	51
PTH	Correlation Coefficient	.062	0.48	-0.444
	p	0.668	< 0.001	0.001
	N	50	50	50

Table 4: Comparison of serum PTH and creatinine levels of patients divided into two groups according to mean lithium level.

		Mean Lithium Level		Z	p
		< 0.8	>= 0.8		
CRE	N	26	25	2.167	0.03
	Median	.76	.90		
PTH	N	25	25	2.775	0.006
	Median	55.92	79.78		

Table 5: Comparison of the biochemical parameters of the patient and control groups after exclusion of individuals with HBA1C values >5.6 mg/dl using antihypertensive and/or oral antidiabetic drugs.

	Group				
	Patient		Control			
	N	Median	N	Median	Z	p
EGFR	36	96.00	30	108.84	-3.227	0.001
Creatinine	36	.79	30	.65	-3.318	0.001
PTH	36	69.00	30	45.07	-3.310	0.001
25-OH D3	30	9,3	29	18.6	-2.259	0.024
Urine Density	35	1007	28	1005	-3.542	<0.001
Creatinine Urine	34	65.71	28	110.92	-2.716	0.007
Uric Acid	34	5.6	30	4.2	-2.909	0.004
Group		N	Mean	SD	t	p
Serum Calcium (CA)	Patient	36	9.8731	.43532	4.591	< 0.001
	Control	30	9.4367	.31237		

The results we obtained in our study show that there is a significant decrease in the glomerular functions of the patients being treated with lithium. The longer the lithium used, the higher the risk of glomerular pathology. These results are in line with studies reporting that long-term lithium use causes deterioration in glomerular functions [20]. Although the shorter average duration of lithium use in studies reporting contrary results suggests that lithium-related glomerular damage becomes more pronounced in long-term use.

In our study, serum uric acid levels were also found to be significantly higher in patients. This result, which is thought to be caused by the decrease in renal metabolism of uric acid, supports the presence of glomerular damage in patients. As far as we know, there is no study showing a direct relationship between lithium use and hyperuricemia. It can be thought that the high serum uric acid levels obtained in our study may be related to the decreased glomerular filtration rate.

While proteinuria was observed in 4 people in the patient group, proteinuria was not detected in the control group, but there was no statistical difference between the two groups. eGFR and serum creatinine levels were within normal limits in 3 of 4 patients with proteinuria. Tubular proteinuria may be seen in the course of chronic tubulointerstitial changes due to lithium. However, since the subtype of this proteinuria could not be determined in our study, it was not possible to make a further comment. Significant proteinuria (>2 g/day) is an indicator of glomerular damage [21]. It is not possible to detect a glomerular pathology with a single measurement, since false positive results may be seen. For this reason, repeating the examination to increase reliability is recommended. Another important point is the relationship between proteinuria and urine density. For example, proteinuria when urine density is 1,030 (++) may not be significant, whereas proteinuria when urine density is 1,005 (+) may reflect a severe proteinuria [22]. A daily protein loss

of <150 mg/day in the urine is considered physiological. Calculation of the total protein/creatinine ratio (mg/mg) in spot urine has been shown to reflect daily protein excretion. It is also unaffected by urine concentration and volume [23]. In terms of a glomerular pathology, we think that it would be more informative to examine the total protein/creatinine ratio and microalbuminuria in addition to proteinuria in the spot urine.

Since additional diseases such as hypertension and diabetes mellitus may impair glomerular functions, statistical analysis was performed again, excluding people with diseases that may affect glomerular functions other than lithium use. In the analysis performed with 36 patients and 30 control groups, excluding individuals using oral antidiabetic and/or antihypertensive drugs and have HBA1C levels>5.6 mg/dl from both groups, eGFR levels were found to be significantly lower and serum creatinine and uric acid levels were found to be significantly higher in patients. Despite the exclusion of factors other than lithium in the patient group, significant loss of glomerular function indicates that lithium use alone is an important risk factor. However, in clinical practice, it is important to prevent and treat diabetes and hypertension, which cause renal dysfunction in patients using lithium.

In our study, eGFR was found below the critical limit in 8 (15.68 %) of 51 patients and required further nephrological evaluation. Some studies also suggest that lithium intoxication episodes may pose a risk for long-term renal failure [24]. It is known that a history of lithium intoxication is more common in patients with renal failure, it is also stated that a slow but increasing rise in creatinine levels may also be a messenger of lithium intoxication [13]. In a recent study, it was determined that a single lithium blood level value measured above 1 mmol/L causes a significant acute decrease in the GFR levels of patients. It was emphasized that it is not known whether these acute decreases in renal functions observed in this patient group are compensated in the long term, and that lithium blood level measurements should be performed at least every 3 months [25]. Similarly, in our study patients with serum lithium levels above 0.8 mEq/L in the last year had higher serum creatinine levels compared to patients with low lithium levels, which is an important finding showing the disruptive effect of lithium. In a study, the kidney functions of 77 patients with a history of lithium intoxication were examined. It was found that the serum creatinine values before intoxication did not differ significantly from the serum creatinine values measured at least one month after the intoxication, and the increased creatinine levels returned to normal levels after a short time [26]. There is not enough information in the literature about the extent to which lithium intoxication attacks affect kidney functions in the long term. In our study, 13 patients (25.49 %) had a history of lithium intoxication, and serum creatinine, GFR and uric acid parameters reflecting glomerular functions of these patients did not show a significant difference compared to the patient group without a history of intoxication. Although this suggests that acute changes in kidney functions in patients with lithium intoxication may improve to a certain extent in the long term, studies investigating the long-term effects of lithium intoxication are needed.

4.2. Findings Related to Tubular Functions

Impaired urinary concentration ability is the most common renal side effect observed due to lithium treatment. The incidence of nephrogenic diabetes insipidus (NDI) among patients treated with lithium ranges from 20 % to 87 % [15]. In our study, urine density showing tubular functions was found to be significantly lower in the patient group and this is consistent with the literature.

4.3. Findings Related to Calcium Metabolism

One of the studies investigating whether there is a correlation between long-term lithium use and calcium metabolism has reported that the risk of developing hypercalcemia in patients using lithium for 15 years is 3 to 6 times higher than the normal population [27]. Similarly, calcium and PTH levels were found to be significantly higher in the patient group in our study. While hypercalcemia was detected in 6 people in the patient group, it was not detected in the control group. In another study, PTH and calcium values of 31 patients who were started on lithium treatment were compared with measurements after an average of 18 months of follow-up. It was found that PTH levels increased significantly during lithium treatment, but there was no significant change in ionized and total calcium levels. It was found that 5 of 31 patients whose PTH levels were within the reference range before follow-up developed hyperparathyroidism after follow-up [28]. In another study, 18 % of 423 patients in whom the prevalence of lithium-related hyperparathyroidism was attempted to be determined had lithium-related hyperparathyroidism, and 43 % had vitamin D insufficiency [29]. It is known that vitamin D deficiency can cause elevations in PTH levels [30]. In our study, 25-OH D3 levels were found to be significantly lower in the patient group. However, no significant relationship was found between PTH levels and 25-OH D3 levels. This result suggested that lithium use affects the functions of the parathyroid gland independently of vitamin D levels. Higher PTH and calcium levels in the patient group indicate a higher risk in terms of cardiac diseases, osteoporosis and renal diseases in the patient group using lithium. This situation requires clinicians to be more careful about these side effects.

5. Conclusion

Our study was conducted in a specialized mood outpatient clinic in the patient group with regular prospective follow-up and long-term lithium use, and it was found that the parameters indicating kidney functions such as creatinine, uric acid, calcium, PTH levels were higher, eGFR, Vitamin D, urine density

were lower than the controls, and in fact, about one of the 7 patients (n=8/51, 15.68 %) deteriorated critically, one quarter of the patients had a toxic level of lithium, and this situation was associated with impaired renal function and high PTH levels, duration of lithium use was moderately correlated with uric acid, and creatinine levels, and eGFR. Considering that even though the treatment was terminated, the glomerular pathology progressed irreversibly after a point, so the need for careful monitoring of the kidney functions of the patients receiving lithium conservation therapy was re-emerged in this study. The results reveal the usefulness of investigating proteinuria, total protein/creatinine ratio, and microalbuminuria in the urine, apart from serum markers, for a glomerular pathology. Before starting lithium conservation therapy, renal functions, especially eGFR, should be evaluated and repeated every 6 months, and lithium blood levels should be checked at least every 3 months. According to the results of the research, it can be recommended to continue the treatment of the patients with the lowest possible therapeutic blood levels, to determine the response level to lithium in the clinical follow-up, and to discontinue lithium in patients who do not benefit from the treatment. Since there are still gaps in the mechanism and process of lithium nephrotoxicity, studies with large sample sizes and long follow-up periods are needed.

Limitations of the Study

This study has some limitations, such as the relatively small number of cases, the inability to completely exclude other factors that may affect kidney functions other than lithium use, and due to sample having patients using lithium a moderate or longer period, the lack of data on patients who used lithium for a much longer period of time, the lack of data on patients who have used lithium for a long time and discontinued. In addition, the cross-sectional design of our study creates some limitations in establishing a causal relationship. Nevertheless, the strength of the study is that the patients were a carefully selected and regularly monitored cohort in a specialized mood outpatient clinic.

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Conflict of Interests

The authors declare no conflict of interest.

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Author Contributions

Conceived and designed the analysis: SÇ, BA, HY. Collected the data: BA, RT. Contributed data or analysis tools: BA, RT. Performed the analysis: BA, RT. Wrote the paper: BA, RT. Language editing: SÇ, HY. Final approval: SÇ, HY.

Ethical Approval

The protocol of the study was approved by Istanbul University Non-Interventional Clinical Research Ethics Committee (File no: 2015/1999).

Data sharing statement

All data relevant to the study are included in the article.

Consent to participate

All participants read the consent form and understand the study being described.

Informed Consent

Written informed consent was obtained from all patients at the of the study.

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Brucellosis, a Rare Cause of Muscle and Joint Pain Following Covid-19 Treatment in Endemic Regions

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Abstract: In our area, where endemic Brucella is present, we intended to demonstrate the frequency of Brucella among patients who applied malaise, muscle, and joint pain following Covid-19 treatment in the Long Covid period. In this study, 86 patients who were PCR positive between April 2020 and May 2022, diagnosed with Covid-19, and completed their treatment, were retrospectively analyzed. Thirteen patients with ongoing complaints of brucella agglutination and/or brucella coombs agglutination of 1/160 and above were included in the study. Five (38 %) patients in the study were male (31 to 69, median age 55 years), and eight (62 %) were female (40 to 57, median age 43.5). Muscular-joint pain (62 %), malaise (62 %), fatigue (30 %), and sweating (15 %) were common symptoms. The duration of onset of symptoms in the Long Covid period ranged from 41 days to 220 days. Brucellosis should be considered in the differential diagnosis of patients diagnosed with Covid-19 in endemic regions ad presenting to the hospital with symptoms of muscle-joint pain and malaise after treatment. © 2023 NTMS.

KeyWords: Brucella; Covid-19; Muscle-Joint Pain.

1. Introduction

Covid-19 infection was first described in the Chinese city of Wuhan in the province of Hubei on 12 December 2019 [1]. The World Health Organization (WHO) declared it a pandemic on 11 March 2020 [2, 3]. Symptoms generally associated with Covid-19 include fever, cough, respiratory difficulty, headache, and muscle-joint pain [4].

Muscle-joint pain, malaise, and fever can persist in the Long Covid period [5]. In NICE guidelines: Acute Covid-19: Symptoms up to 4 weeks, ongoing symptomatic Covid-19: Symptoms between 4 and 12 weeks, post-Covid-19: Symptoms that develop after 12 weeks and persist for longer [6]. The term "Long Covid" includes both post-Covid and ongoing symptomatic Covid

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syndrome, according to NICE guidance [6]. Symptoms such as muscle-joint pain, weakness, and fever that continue in the Long Covid period are not specific. In some studies, similar symptoms can be seen in the underlying autoimmune disease, chronic fatigue syndrome, and post-chikungunya syndrome, a seronegative disease that progresses with an insufficient inflammatory response in endemic areas [5].
Patients with brucellosis generally present to the hospital with symptoms such as fever, shivering, fatigue, and diffuse muscle joint and back pain [7]. Because brucellosis symptoms are similar to Long Covid symptoms, it may be confused and even the diagnosis of brucellosis may be missed. Since there is no study on brucellosis, we aimed to present the patients who were diagnosed with brucella after presenting with Long Covid symptoms in areas where brucella is endemic.

2. Material and Methods

This retrospective study was performed in Adıyaman University Faculty of Medicine Infectious Diseases Clinic. In this retrospective study, 86 patients with Covid-19 PCR (+) between 01.04.2020 and 01.05.2021 were retrospectively analyzed. Brucella agg. test 1/160 and, brucella coombs agg. patients with (+) (n=13) were included. Although the pain continues, brucella Agg. test 1/160 and brucella coombs agg. Patients with (–) (n=73) were excluded.
Inclusion criteria for the study; history of Covid-19 infection, Covid-19 PCR positivity, over 18 years of age, and symptoms of muscle-joint pain and weakness. Exclusion criteria of patients; Covid-19 PCR negativity at the time of symptoms, being under the age of 18, patients with ongoing Covid-19 treatment and lung involvement, and patients with no regression in Covid-19 symptoms.
Brucella diagnosis was made by serology in the presence of accompanying symptoms. Titers of ≥1/160 were considered positive in the anti-human globulin (Coombs) test. Age, gender, history of Covid-19 disease, duration of symptoms, and serology results of the patients were recorded.

2.1. Statistical Analysis

Data were analyzed on Statistical Package for Social Sciences Statistical Software, version 21.0 (SPSS Inc). Descriptive statistics were produced. Data were expressed as numbers (percentage) and median (minimum-maximum).

3. Results

Brucella was diagnosed in 13 of 86 patients who had ongoing muscle-joint pain, weakness, fatigue, fever, sweating, and headache complaints during the Long covid period. Five (38 %) patients in the study were male (31 to 69, median age 55 years), and eight (62 %) were female (40 to 57, median age 43.5). Muscular-joint pain (62 %), malaise (62 %), fatigue (30 %), and sweating (15 %) were common symptoms. The duration of onset of symptoms in the Long Covid period ranged from 41 days to 220 days.
The onset of symptoms ranges from 41 days to 220 days, with a mean duration of 119 days. The positivity in brucella agg./brucella coombs agg. test ranged from 1/160 to 1/5120. The symptoms and characteristics of the patients included in the study are shown in Table 1. Since the study is retrospective, the study was conducted by considering the laboratory results registered in the system. There are cases of acute and chronic brucellosis among the patients.

Table 1: Patients' symptoms and other characteristics.

Case	Sex	Age	Symptoms	Brucella Agg.	Time Elapsed
1	Female	40	Low Back Pain, Malaise, Fatigue	1/160	92 Days
2	Female	51	Night Sweats, Malaise	1/320	78 Days
3	Female	43	Muscle-Joint Pain	1/320	120 Days
4	Female	54	Malaise, Total Body Pain	1/160	88 Days
5	Male	49	Muscle-Joint Pain	1/5120	183 Days
6	Male	64	Muscle-Joint Pain, Malaise	1/320	136 Days
7	Female	43	Respiratory Difficulty, Fatigue	1/640	106 Days
8	Female	57	Muscle-Joint Pain, Malaise	1/640	94 Days
9	Male	69	Malaise, Headache, Vertigo	1/160	41 Days
10	Male	55	Muscle-Joint Pain, Fatigue	1/640	104 Days
11	Male	31	Muscle-Joint Pain, Malaise	1/160	180 Days
12	Female	42	Muscle-Joint Pain, Fatigue	1/2560	105 Days
13	Female	44	Muscle-Joint Pain, Malaise, Sweating	1/640	220 Days

4. Discussion

Brucellosis was diagnosed in 13 cases with a history of Covid-19 disease, who applied to the hospital with muscle-joint pain, weakness, and fatigue during the Long Covid-19 period. In these cases, brucella agg./brucella coombs agg. tests were positive, varying over 1/160. The patients were diagnosed with brucellosis and treatment was started. We are currently facing an unprecedented epidemic. Uncertainties about the disease continue after (Long Covid).

This means that every detail about the disease needs to be evaluated in detail to remove uncertainties. Available evidence and WHO reports indicate that pain is a common symptom during SARS-CoV-2 infection. Muscle pain, joint pain, sore throat, and headache are the pain-related symptoms seen in Covid-19 [9].

In some cohort studies, muscle pain, arthralgia, and fatigue have been reported in Covid-19 patients [9, 10, 11]. Symptoms of Covid-19 disease may persist even if the disease resolves, and the PCR is negative. Most of the symptoms in the Long Covid period show features similar to those that developed in the acute phase of Covid-19 [12]. These symptoms are usually fever, cough, shortness of breath, headache, muscle-joint pain, and fatigue. One or more of these symptoms continue to be seen during the Long Covid period [4, 13].

Brucellosis is a ubiquitous zoonotic disease widely seen in the Mediterranean region, the Indian subcontinent, the Middle East, Africa, Central America, and Central Asia. The most frequent symptoms of brucellosis are muscle-joint pain, fatigue, back pain, and shivering [14].

Another study showed that 80% of individuals with a confirmed diagnosis of Covid-19 continued to have at least one symptom two weeks after acute infection. Fatigue is the most common of these symptoms in acute Covid-19 and Long Covid periods. Some symptoms may persist even 100 days after the first acute Covid-19 symptom [15]. Brucella spondylodiscitis is a disease with cardiac involvement and cranial involvement [16]. Long covid symptoms in endemic areas should not delay the diagnosis in patients with brucellosis. Times to presentation to hospital in the Long Covid period among the 13 cases in the present study ranged between 41 days and 220 days. We attribute the prolonged time to diagnosis of brucellosis in these patients to the symptoms seen in these patients being ascribed to Long Covid symptoms.

Symptoms seen during the Long Covid period may resemble those of various other diseases. Although clinical examination results and patient symptoms vary, chronic fatigue syndrome is one of the most frequently seen conditions in the Long Covid-19 period. Chronic fatigue syndrome is compatible with dysautonomia [17]. Covid-19 also plays a probable triggering role in the immune system, as in Guillain-Barre syndrome and other autoimmune diseases. A possible underlying autoimmune disease should therefore also be considered in the differential diagnosis of young women presenting with muscle-joint pain in the Long Covid period [12]. In addition, studies have suggested that post-chikungunya syndrome, a seronegative disease progressing with insufficient inflammatory response, should also be considered in the differential diagnosis of patients presenting with high fever, headache myalgia, and diffuse joint pain in the Long Covid period [5, 13].

In this study, A diagnosis of brucellosis was made in 13 patients whose complaints continued after the treatment of Covid-19 and who applied to the hospital. In these cases, brucella agg./brucella coombs agg. tests were found to be 1/160 and above positivity. The patients were diagnosed with brucellosis and treatment was started.

5. Conclusions

Brucellosis is a condition that can lead to more serious complications if its diagnosis is delayed. For this reason, in Long Covid cases, especially in brucella endemic regions, cases with symptoms that may overlap with the brucellosis clinic should be evaluated in terms of brucellosis. This approach will be useful in preventing more serious complications that may develop later.

Limitations of the Study

It is our limitation that it is a retrospective study, and the number of cases is low. Among the other limitations of my study, it could not be distinguished clinically and laboratory, that all the cases may not have been admitted to the hospital, that their covid history may have increased the susceptibility to immune suppression and therefore to brucellosis-like infective conditions. The fact that they were not evaluated for other pain syndromes and that only patients from one clinic were included may have caused possible population bias. Considering these situations, we think that more comprehensive similar studies will make a great contribution to the literature.

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Conflict of Interests

The authors accept their responsibilities in the study. There is no conflict of interest between the authors.

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Author Contributions

SA and HSS designed the study; SA and HSS collected the data; SA ve HSS analyzed the data; SA wrote the first draft of the manuscript. All authors contributed to the study's conception and design. All authors read and approved the final manuscript.

Ethical Approval

This study was approved by the ethics committee of Adıyaman University (No. 2021/03-20).

Data sharing statement

All data relevant to the study are included in the article.

Consent to participate

Written informed consent was obtained from every patient at the time of the operation.

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Investigation of the Effectiveness of the ADA Prediabetes Risk Test in Identifying Prediabetic Turkish Patients and Determination of the Frequency of Retinopathy in Prediabetic Patients

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Abstract: In this study, the Turkish version of the American Diabetes Association (ADA) prediabetes risk test and the 75-g oral glucose tolerance test (OGTT) were administered to patients to determine the rate of prediabetic patients in our geographical region and investigate its consistency with the ADA risk test. In addition, the presence of retinopathy in prediabetic patients was examined. The study included a total of 342 patients with a fasting plasma glucose value of 100-125 mg/dl. The OGTT and risk test results were compared. According to the ADA prediabetes risk test, the patients were classified into those at risk for type 2 diabetes mellitus (DM) and those diagnosed with prediabetes. Prediabetes diagnosis determined by OGTT and HbA1C. When the OGTT results of the patients with negative ADA prediabetes risk test scores were compared, the sensitivity was determined as 54 % and the specificity as 63 % ($p<0.05$). When the patients with positive ADA prediabetes risk test scores were compared with those diagnosed with prediabetes and type 2 DM, the sensitivity and specificity values were calculated as 58 % and 54 %, respectively ($p>0.05$). There was no retinopathy finding in the eye examination of 262 of the 342 patients included in the study ($p>0.05$). In this study, we found that a positive ADA prediabetes risk score was effective in predicting prediabetes, but it was not sufficient. However, prediabetes diagnosed according to OGTT was found to be higher in the patients with negative test scores. © 2023 NTMS.

Keywords: Prediabetes; Type-2 Diabetes; ADA Risk Test; OGTT; Retinopathy.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease in which the organism cannot adequately benefit from carbohydrates, fats, and proteins due to insulin deficiency or defects in the effect of insulin and requires continuous medical care and treatment [1].

Prediabetes is an intermediate state of hyperglycemia with a high risk for type 2 DM. The process between a normal glucose metabolism and overt diabetes is called the 'prediabetic period' [2]. There are three conditions in prediabetes patients: isolated impaired fasting glucose

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(IFG), isolated impaired glucose tolerance (IGT), and combined IFG+IGT. If these three conditions are left untreated, they progress to overt diabetes.

According to some publications, the rate of progression to diabetes in prediabetic patients is 70 %. Therefore, the early diagnosis and prevention of DM development and related clinical complications increase the clinical importance of the disease [3]. Observational studies have reported that prediabetes is associated with nephropathy, small fiber neuropathy, retinopathy, chronic kidney disease, and increased risk of cerebrovascular and cardiovascular diseases. The main purpose of treatment in prediabetes is to prevent the development of diabetes. An easy, practical, and cost-effective way to identify individuals at risk for prediabetes and diabetes is the American Diabetes Association (ADA) prediabetes risk test.

Diabetic retinopathy is the most common preventable or treatable chronic microvascular complication of DM. In other words, it constitutes the most common cause of preventable and/or treatable blindness in adults aged 20-74 years [4]. However, data on the development of retinopathy in prediabetes are very limited, and therefore the relationship between prediabetes and retinopathy needs further investigation. Treatment methods to be applied in retinopathy detected in the early period can prevent the development of advanced retinopathy, maculopathy, and blindness.

This study aimed to determine the rate of prediabetic cases among the patients that presented to the internal medicine outpatient clinic with a suspected risk of prediabetes, evaluate their ADA risk test scores, and investigate the relationship between prediabetes and retinopathy in these patients.

2. Material and Methods

This study was conducted prospectively at the Endocrinology and Metabolic Diseases Outpatients Clinic and Internal Medicine Outpatient Clinic of Erzurum Regional Training and Research Hospital between June 2016 and December 2017. The study was commenced after receiving approval from the local ethics committee (ethics committee date: 19.02.2018, number: 37732058-514.10). A total of 342 patients with a fasting plasma glucose (FPG) value of 100-125 mg/dl and 75-g oral glucose tolerance test (OGTT) results were included in the study. The patients were informed, and their consent was obtained. The ADA prediabetes risk test was administered to the patients, and the related data were noted. Height, weight, and waist circumference measurements were made. The laboratory results of the patients were accessed and noted through the hospital's automation system. Patients with known eye diseases, such as glaucoma and a history of eye surgery were not included in the study.

The laboratory procedures of the study were carried out in the biochemistry laboratory of the hospital. Venous blood was drawn from the antecubital vein after the patients fasted for 12 hours. The patients' gender, age, diagnosis, FPG value at the time of diagnosis, 75-g OGTT result, HbA1C, uric acid, total cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and basic biochemical tests were recorded from the hospital automation system and patient files.

Among the anthropometric measurements of the patients, body weight was determined with the patient wearing the thinnest clothes possible and barefoot. Height was measured with the patient standing bare feet and his/her feet positioned together, while leaning perpendicular to the height measurement ruler. Waist circumference was measured with a tape from the midpoint of the distance between the lowest rib and the iliac crest. Body mass index (BMI) was calculated by dividing the body weight (kg) by the square of the height in meters (kg/m^2).

The ADA prediabetes risk test includes seven simple and easy-to-understand questions about age, gender, history of gestational diabetes, family history of DM, high blood pressure, physical activity, and height-weight. Scores of 5 and above in this test are accepted as positive and indicate risk for type 2 DM.

After 10-12 hours of fasting, 75-g OGTT was performed on the patients, and their HbA1C levels were measured. Before the test, FPG was evaluated, and plasma glucose (PG) was measured in the second hour after the patients ingested 75 g of oral glucose solution. Patients with an FPG value 100-125 mg/dl and second-hour PG of <140 mg/dl were evaluated as having IFG, those with an FPG of <100 mg/dl and second-hour PG of 140-199 mg/dl as having IGT, those with an FPG of 100-125 mg/dl and second-hour PG of 140-199 mg/dl as having combined IFG+IGT, those with a second-hour PG of >200mg/dl as diabetic, and those with an FPG of <100 mg/dl and second-hour PG of <140 mg/dl as having normal values.

According to these results, the patients who met one of the following criteria were considered prediabetic.

1.HBA1C of 5.7-6.4 %

2.FPG of 100-125 mg/dl

3.Second-hour PG of 140-199 mg/dl in OGTT.

2.1. Retinopathy assessment

Each patient underwent a complete ophthalmologic examination, including visual acuity, intraocular pressure measurement (Goldmann applanation), anterior segment and fundus examinations, and central foveal thickness (CFT) and mean foveal thickness (MFT) measurements. CFT and MFT measurements were undertaken using spectral-domain optical coherence tomography (SD-OCT) (Optovue Inc., Fremont, CA, USA) in a dim room by instilling a 0.5 % drop following tropicamide mydriasis. All the SD-OCT evaluations were performed by a single ophthalmologist. The mean outcome measures, CFT, and MFT were automatically calculated by SD-OCT.

2.2. Statistical Analysis

Data were presented as mean ± standard deviation, percentage, and number. The analysis of the research data was undertaken using the Statistical Package for the Social Sciences (SPSS) for Windows, v. 17.0. The normality of the distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test and histograms. In the comparisons between two independent groups, the independent-samples t-test was used when the normal distribution condition was met. Comparisons between categorical variables were made with the chi-square and Fisher's exact tests. In the comparison of two continuous variables, the Pearson test was conducted if the normal distribution condition was met and the Spearman correlation test otherwise. The receiver operating characteristics (ROC) analysis was performed to determine whether the continuous variable could be used in the diagnosis. The results were evaluated at the 95 % confidence interval by taking the statistical significance level as $p<0.05$.

3. Results

The study included a total of 342 patients, of whom 34.2 % (n=117) were male and 65.8 % (n=225) were female. The mean age of the patients was 53.1 [standard deviation (sd): 12.5] years. The lowest patient age was 18 years and the highest was 85 years. The mean age of the male patients was 55.1 years, and that of the female patients was 52.1 years. Other demographic and laboratory parameters of the patients are detailed in Table 1.

The prediabetes diagnosis of the patients included in the study was made based on the OGTT and HbA1C results. Accordingly, 95.2 % of the patients whose FPG value was 100 mg/dl and above were diagnosed with prediabetes. In the remaining 4.8 % of the patients, both the OGTT and HbA1C results were found to be normal. According to the ADA prediabetes risk test, the rate of patients with type 2 DM risk was 72.5 % in the group diagnosed with prediabetes and 37.5 % in the group without prediabetes. Accordingly, among the patients with prediabetes, the risk of type 2 DM was found to be significantly higher than in those without prediabetes (p=0.001) (Table 2).

When the patients were examined according to the diagnosis of prediabetes, it was found that 16 did not have prediabetes. When these 16 patients were further examined according to their scores in the prediabetes risk test, 53.3 % (n=8) had 4 points, 33.3 % (n=5) had 5 points and the remaining 6.66 % (n=1) had 2 points, i.e., the majority of those without prediabetes scored 4 or 5.

The patients with a score of 5 and above in the prediabetes risk test were considered to be at risk for type 2 DM. When examined from this perspective, it was determined that 70.8 % of the patients were at risk. There was no significant difference between the male and female patients in terms of prediabetes risk ($p>0.05$). The mean scores of the patients in the prediabetes risk test was 5.35 (sd: 1.59). It was observed that 79.6 % of the patients had a score between 4 and 7 points.

The OGTT results were normal in only 41 (46.6 %) of the 88 patients who had a prediabetes risk test score of 4 and below, i.e., who did not have type 2 DM risk according to the test. Of these cases, 16 (18.2 %) were classified as IFG, nine (10.2 %) as IGT, 15 (17 %) as combined IFG+IGT, and seven (8 %) as DM. Detailed data are given in Table 3.

According to the OGTT results of the 223 patients with a prediabetes risk test score of 5 and above, i.e., those with type 2 DM risk according to the test, 72 (32.3 %) were evaluated as having normal values, 45 (20.2 %) as having IFG, 24 (10.8 %) as having IGT, 43 (19.3 %) as having combined IFG+IGT, and 39 (17.5 %) as having DM. There was no significant difference between the groups ($p>0.05$) (Table 4).

In the prediabetes risk test, the patients were analyzed in detail according to whether they were at risk of type 2 DM. In the examination made in terms of age, the mean age of those with type 2 DM risk was determined to be significantly higher (p=0.001). The mean age of the patients at risk for type 2 DM was 57.8 years, while the mean age of those without this risk 42 years. The detailed data of the patients with and without type 2 DM risk are given in Table 5.

When the relationship between the HbA1C results and the prediabetes risk test scores was examined, there was an increase in the HbA1C value as the prediabetes risk test score increased. The patients who had a score of 5 and above in the prediabetes risk test, i.e., those that were considered to be at risk for type 2 DM, were found to have a mean HbA1C value above 6 %, while the mean HbA1C value of those with a score of 4 or less in the test was determined to be below 6 % (Table 6).

The patients were divided into two groups according to whether their OGTT results were normal or abnormal. The sensitivity of the prediabetes risk test score in predicting the OGTT outcome was tested using the ROC analysis. In this analysis, the area under the curve was calculated as 0.593, sensitivity as 54 %, and specificity as 63 % ($p =0.001$), suggesting that the ADA prediabetes risk test could be used to identify patients without prediabetes or type 2 DM risk at a statistically significant level (Figure 1).

There was no evidence of retinopathy in the eye examination of 262 of the 342 patients included in the study. The rate of retinopathy in the prediabetic patients was not statistically significant when compared to the diabetic patients ($p>0.05$).

4. Discussion

In this study, the Turkish version of the ADA type 2 prediabetes risk test scores and the 75-g OGTT results were compared. The ADA risk test is a short and easy-to-understand tool that includes seven questions which can be answered by patients themselves. Despite being such a simple and inexpensive test, it is very effective in detecting prediabetes.

Table 1: Demographic and laboratory parameters of the patients by gender.

	Male (n=117, 34.2 %)	Female (n=225, 65.8 %)	Total (n=342, 100 %)
Age	55.10±12.28	52.13±12.53	53.14±12.50
Body Mass Index (kg/m^2)	28.32±4.66	33.04±6.53	31.39±6.35
Creatinine (mg/dl)	0.90±0.16	0.73±0.11	0.79±0.15
Uric acid (mg/dl)	6.04±1.44	5.07±1.27	5.40±1.40
Total cholesterol (mg/dl)	200.26±41.64	206.29±39.52	204.36±40.24
LDL (mg/dl)	127.25±34.25	129.44±31.32	128.68±32.34
HDL (mg/dl)	43.62±10.71	49.04±10.62	47.30±10.93
Triglyceride (mg/dl)	169.27±89.61	153.04±75.69	158.72±81.08
Presence of HT	27.6 % (n=32)	45.2 % (n=98)	38 % (n=130)
Presence of type 2 DM according to the ADA risk test (score 5 and above)	75 % (n=87)	68.5 % (n=148)	70.8 % (n=235)
Mean score in ADA prediabetes risk test (points)	5.50±1.59	5.26±1.59	5.35±1.59
HbA1C (%)	6.03±0.42	6.10±0.40	6.07±0.41

sd: standard deviation, HDL: high density lipoprotein, LDL: low density lipoprotein, HT: Hypertension, DM: Diyabets Mellitus.

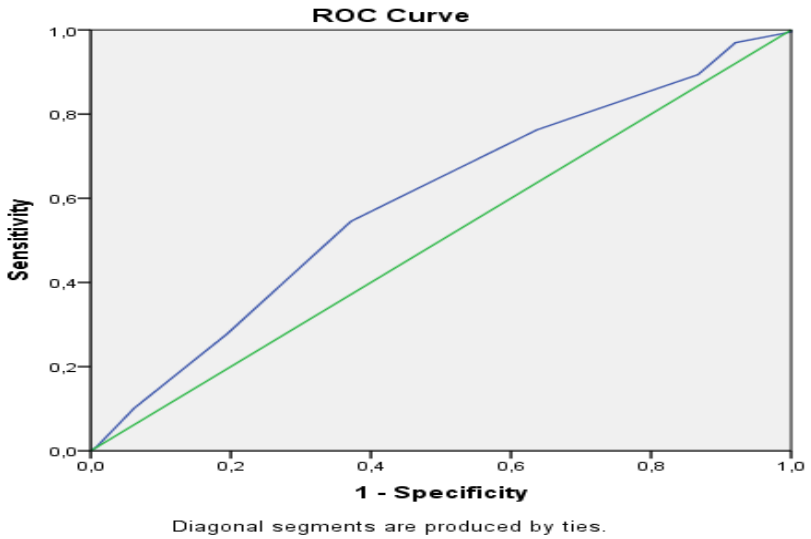

Figure 1: Ability of the ADA prediabetes risk test score to predict normal and abnormal OGTT results in patients.

Table 2: Comparison of the patients with and non-prediabetic.

	Prediabetic (n=316, 95.2 %)	Non-prediabetic (n=16, 4.8 %)	p* value
Age	53.90±11.88	44.25±17.09	**0.001**
Presence of type 2 DM risk	72.5 % (n: 229)	37.5 % (n: 6)	**0.001**

*: Chi-square test, sd: standart sapma, DM: Diyabets Mellitus.

Table 1: OGTT results of the patients with a prediabetes risk score of 4 and below according to the ADA risk test.

	Normal	IFG	IGT	IFG + IGT	DM	Total
1 point	0 (0 %)	1 (100 %)	0 (0 %)	0 (0 %)	0 (0%)	1 (100 %)
2 points	9 (64.3 %)	0 (0 %)	2 (14.3 %)	0 (0 %)	3 (21.4 %)	14 (100 %)
3 points	6 (28.6 %)	5 (23.8 %)	5 (23.8 %)	5 (23.8%)	0 (0 %)	21 (100 %)
4 points	26 (50 %)	10 (19.2 %)	2 (3.8 %)	10 (19.2 %)	4 (7.7 %)	52 (100 %)
Total	41 (46.6 %)	16 (18.2 %)	9 (10.2 %)	15 (17 %)	7 (8 %)	88 (100 %)

Table 4: OGTT results of the patients with a prediabetes risk score of 5 and above according to the ADA risk test.

	Normal	IFG	IGT	IFG + IGT	DM	Total
5 points	30 (41.1 %)	13 (0 %) 17.8	7 (9.6 %)	11 (15.1 %)	12 (16.4 %)	73 (100 %)
6 points	20 (27.4 %)	18 (24.7 %)	9 (12.3 %)	12 (16.4 %)	14 (19.2 %)	73 (100 %)
7 points	15 (30 %)	10 (20 %)	6 (12 %)	11 (22 %)	8 (16 %)	50 (100 %)
8 points	6 (25 %)	2 (8.3 %)	2 (8.3 %)	9 (37.5 %)	5 (20.8 %)	24 (100 %)
9 points	1 (33.3 %)	2 (66.7 %)	0 (0 %)	0 (0 %)	0 (0 %)	3 (100 %)
Total	72 (32.3 %)	45 (20.2 %)	24 (10.8 %)	43 (19.3 %)	39 (17.5 %)	223 (100 %)

Table 5: Mean values of variables and analysis results of groups with and without type 2 DM risk according to the ADA risk test.

	Patients with type 2 DM risk	Patients without type 2 DM risk	p value
Age	57.84±10.44	42.00±9.99	**0.001***
BMI (kg/m^2)	32.52±6.37	28.66±5.42	**0.001***
Creatinine (mg/dl)	0.81±0.15	0.76±0.14	**0.001***
Uric acid (mg/dl)	5.56±1.43	4.99±1.27	**0.001***
HbA1C (%)	6.12±0.39	5.92±0.38	**0.001***
Presence of HT	51.1 % (n=120)	9.3 % (n=9)	**0.001****
Presence of overt DM	35.1 % (n=39)	14.6 % (n=7)	**0.001****

*: Independent-samples t-test, **: Chi-square test BMI: Body Mass Index, HT: Hypertension, DM: Diyabets Mellitus.

Table 6: Comparison of the HbA1C results and ADA prediabetes risk test scores.

Score	Number	Mean HbA1C	Standard deviation
1 point	1	5.90	-
2 points	14	5.90	0.26
3 points	21	5.90	0.39
4 points	53	5.93	0.41
5 points	76	6.01	0.33
6 points	74	6.21	0.52
7 points	53	6.13	0.30
8 points	23	6.19	0.18
9 points	4	6.17	0.20
Total	319	6.07	0.40

This study is one of the few studies using the ADA risk test and OGTT to identify patients at risk of prediabetes and the first research conducted with a Turkish population. We determined that a positive ADA risk score was effective in predicting prediabetes, but it was not sufficient. However, among the patients with negative risk scores, prediabetes was found at higher rates based on the OGTT results. Therefore, the risk test was not effective in predicting prediabetes in those with negative risk scores.

In our study, when the patients' type 2 DM risk in the ADA prediabetes risk test was examined according to their age, it was determined that the mean age of those with type 2 DM risk was 57.8 years, and the mean age of those without this risk was 42 years, indicating a statistically significantly higher value for the former. This finding is consistent with the literature data reporting that the prevalence of diabetes increases with age. According to the IDF 2017 data, the prevalence of prediabetes was the lowest in the youngest patient group and the highest in the oldest patient group [5].

We also investigated whether being at risk for type 2 DM according to the ADA risk test resulted in a significant difference in HbA1C values. The mean HbA1C value was determined as 5.92 % (sd: 0.38) for the patients without type 2 diabetes risk and 6.12 % (sd: 0.39) for those with this risk. The difference between the two groups was statistically significant, and those with type 2 diabetes risk had higher HbA1C values. When the relationship between the HbA1C value and the prediabetes risk test score was examined using the correlation analysis, it was observed that as the prediabetes risk test score increased, the HbA1C value also increased, and there was a statistically significant positive correlation. The patients with a prediabetes test score of 5 and above, i.e., those considered to be at risk

for type 2 DM, were found to have a mean HbA1C value above 6%, while the patients that scored 4 and below in the prediabetes risk test had a mean HbA1C value below 6 %.

There was also a statistically significant difference between the OGTT groups in relation to the HbA1C values. In the subgroup analysis, differences were at significant levels in the pairwise comparisons of the normal and combined IFG+IGT groups, normal and DM groups, IFG and combined IFG+IGT) groups, IFG and DM groups, IGT and DM groups, and DM and combined IFG+IGT groups. The mean HbA1C value of the DM group was found to be statistically significantly higher compared to the remaining groups. In addition, the mean HbA1C value of the combined IFG+IGT group was statistically significantly higher compared to the normal and IGT groups.

Türkiye Diyabetes, Obesity and Hypertension Epidemiology 2 and various studies have revealed that the high-risk group determined by HbA1C includes people with more severe glucose metabolism disorders than those with isolated IFG and isolated IGT and close to those with IFG+IGT [6]. It has been reported that individuals that are determined to be at high risk of diabetes according to the A1C test performed with a standard method are more likely to develop this disease, and therefore they should be included in diabetes prevention studies [1].

In another study examining the data obtained from seven studies investigating HbA1C in diabetic cases, the incidence of diabetes over a five-year follow-up was reported to be <5 % for the HbA1C range of 5.0-5.5 %, 9-25 % for the HbA1C range of 5.5-6.0 %, and 25-50 % for the HbA1C range of 6.0 and 6.5 % [7]. Similarly, in our study, the risk of developing diabetes was found to be higher as the HbA1C level increased.

In the current study, according to the OGTT results of the 223 patients with a prediabetes risk test score of 5 and above, i.e., those with type 2 DM risk, 72 (32.3 %) had normal values, 45 (20.2 %) had IFG, 24 (10.8 %) had IGT, 43 (19.3 %) had combined IFG+IGT, and 39 (17.5 %) had DM. There was no significant difference between the groups.

Only 41 (46.6 %) of the 88 patients with a prediabetes risk test score of 4 and below, i.e., those without type 2 DM risk, had normal OGTT results. Sixteen (18.2 %) of these cases were classified as IFG, nine (10.2 %) as IGT, 15 (17 %) as combined IFG+IGT, and seven (8 %) as DM based on the OGTT results. According to OGTT, we also evaluated the patients in two groups as normal and abnormal results. We performed the ROC analysis between the OGTT results of the patients with negative scores in the prediabetes risk test and the prediabetes risk scores. In the ROC analysis, the AUC, sensitivity, and specificity values were determined to be 0.593, 54 %, and 63 %, respectively, suggesting that the ADA prediabetes risk test could be used to identify patients without prediabetes and type 2 DM risk at a statistically significant level. We also observed that the patients with positive risk scores had normal OGTT results. Therefore, the use of the ADA risk test is helpful but needs confirmation by another test.

The Finnish Diabetes Risk Score (FINDRISC) is another scoring system developed to identify individuals at risk for diabetes through simple and easy-to-understand questions scored in a similar manner to the ADA test. Among the studies in the literature using this scoring system, Vandersmissen et al., reported that FINDRISC was useful in identifying patients at risk of diabetes [8]. In a study by Kutlu et al., including 479 patients from the Turkish population, a correlation was found between the FINDRISC survey results and FBG levels [9]. Martin et al. compared the HbA1C, FINDRISC, and OGTT results to investigate diabetes risk in healthy individuals and determined that the FINDRISC test was an inexpensive, reproducible, and reliable method for this purpose [10].

In the literature, there are many similar studies using FINDRISC, but there is no large-scale study for the Turkish population. In our review of the literature on the ADA risk test, a scoring system similar to FINDRISC, we did not find satisfactory results. In a study by Tentolouris et al., random capillary glucose, ADA diabetes risk test, and skin fluorescence spectroscope results were compared. On completion of the study, the authors observed that the ADA risk test was significant in identifying both prediabetic and diabetic patients diagnosed based on their HbA1C values [11]. When all the literature findings are considered together, it can be concluded that there are not a sufficient number of studies on the ADA risk test. Our study is one of the few conducted to evaluate the effectiveness of the ADA risk test in identifying patients at risk of diabetes with respect to OGTT results, and it is also the first study undertaken with a Turkish population.

Of patients diagnosed with type 2 DM, 10-40% have complications at the time of diagnosis. Therefore, it should be considered that prediabetes is not a silent stage but contains the health risks of diabetes. This period may cause a series of problems in terms of the development of not only microvascular and macrovascular diseases but also other health risks [12].

Diabetic retinopathy is the most common preventable or treatable chronic microvascular complication of diabetes. In other words, it is the most common cause of preventable and/or treatable blindness in adults aged 20-74 years [4]. However, data on the development of retinopathy in prediabetes are very limited. There is still no large-scale study in the literature investigating the relationship between prediabetes and retinopathy in Turkey [12]. Almost all cases of diabetic retinopathy among prediabetic patients have been reported to be mild non-proliferative diabetic retinopathy [12]. To our knowledge, our study is the first to investigate retinopathy in prediabetic patients in the Turkish population.

In the DPP study, in which 878 prediabetic patients were followed up for three years, diabetic retinopathy was detected by fundus photography in 12.6 % of the

594 patients with diabetes and 7.9 % of those without diabetes [13]. In a meta-analysis covering 12 studies on retinopathy, the data of 44.623 patients were evaluated, and the prevalence of total retinopathy was determined as 6.7 %, and the prevalence of DM-specific retinopathy was 1.5 %. Furthermore, the prevalence of diabetic retinopathy was 9.4 % in individuals with known DM, 1 % in the presence of newly diagnosed DM, 0.1 % in the presence of IGT, and 0.1 % in the presence of IFG [14-15]. In our study, however, retinopathy was not detected in the presence of prediabetes. Although retinopathy was found in 0.1 % of prediabetic cases in the meta-analysis, the relationship between prediabetes and retinopathy can be revealed more clearly by increasing the number of patients in our study. In studies included in the meta-analysis, diabetic retinopathy was classified according to the retinal photographing method. We consider that if we had used retinal photographing method in our study, we could have found a significant relationship between prediabetes and retinopathy.

5. Conclusion

In this study, it was determined that a positive ADA risk test score was effective in predicting prediabetes, but it was not sufficient. The diagnosis of prediabetes based on the OGTT results was found to be higher in patients with negative ADA risk scores. However, prediabetes diagnosed according to OGTT was found to be higher in the patients with negative scores. Therefore, the prediabetes risk test was not effective in predicting prediabetes among the patients with negative scores.

Limitations of the Study

The major limitation of our study is that it only included patients who presented to the outpatient clinic of a single center, which reduces the generalizability of the findings to the whole Turkish population.

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Conflict of Interests

The authors declare no conflict of interest.

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Author Contributions

FC contributed to the writing-original draft preparation of the manuscript, writing-review & editing, methodology, visualization, and investigation. AVŞ contributed to conceptualization, writing-review & editing, and data curation. İB contributed to formal analysis, resources, and visualization. DNB contributed to investigation, software, and resources. Final approval was given by FC, AVŞ, İB, and DNB.

Ethical Approval

The study was commenced after receiving approval from the local ethics committee (ethics committee date: 19.02.2018, number: 37732058-514.10).

Data sharing statement

None.

Consent to participate

Informed consent was obtained from the patients.

Informed Consent

The study complies with the principles of the Declaration of Helsinki. The consent of all the patients was obtained before commencing the study.

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