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Original Article

ANALYTICAL METHOD VALIDATION AND BIOEQUIVALENCE STUDY OF ERLOTINIB 150 MG TABLETS IN IRANIAN HEALTHY VOLUNTEERS UNDER FASTING CONDITION

GHASEMIAN ELHAM[1], SADRAI SIMA[2], SHOKRI JAVAD[3], ILKA A.[4]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Islamic Azad University of Damghan, Damghan, Iran, [2]Division of Biopharmaceutics and Pharmacokinetics, Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, [3]Pharmaceutical Department, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran, [4]Molecular Biology Department, Islamic Azad University, Tehran North Branch, Tehran, Iran

***Email: ghasemian_elham@yahoo.com**

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ABSTRACT

Objective: This study aims to compare a generic formulation of the drug erlotinib 150 mg tablet to the brand-name version to validate the analytical method and bioequivalence studies.

Methods: Erlotinib hydrochloride tablets (test versus reference formulation) were compared in a randomized, two-period crossover study to determine their pharmacokinetic properties and bioequivalence in healthy Iranian volunteers. 14 d passed between each treatment during the washout period. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to analyze erlotinib, and the method validation is presented.

Results: Over the range of 6.25-3200 ng/ml, the analytical method was verified as linear (R2= 0.998). The technique was also accurate and precise at various concentrations. The results showed that the pharmacokinetics of the two products were comparable. Following administration of the test and reference products, the geometric averages for (Area under the curve) AUC_{0-72}, AUC_{inf}, and maximum plasma concentration (C_{max}) were 104.71 (90% CI, 93.39-117.40), 104.68 (90% CI, 93.47-117.23), and 104.85 (90% CI, 94.61-116.21), respectively. The outcomes fell within the permitted tolerance of 0.8 to 1.25.

Conclusion: For the determination of erlotinib in plasma, the used analytical approach is accurate, precise, repeatable, and selective. Additionally, the bioequivalence research revealed no appreciable differences in pharmacokinetic characteristics between the reference and test products. Therefore, it is possible to assert that the generic erlotinib product and the reference product are bioequivalent.

Keywords: Bioequivalence, Pharmacokinetics, Lung cancer, Erlotinib, LC-MS/MS

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INTRODUCTION

The primary reason for cancer-related deaths globally is non-small cell lung cancer (NSCLC) [1]. In 2004, the FDA approved erlotinib (Tarceva®; OSI Pharmaceuticals and Genentech) as a monotherapy for the treatment of patients with locally advanced or metastatic NSCLC following the failure of at least one prior chemotherapy regimen [2]. Erlotinib acts as a potent and highly selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase [3].

Erlotinib has a low water solubility (0.4 mg/ml at pH 2) and is a lipophilic medication. Because of its slow dissolution rate, it has low absorption and bioavailability; oral dosing demonstrated approximately 60% bioavailability when administered fasted and up to 100% bioavailability when administered with food [4, 5]. Peak plasma levels following an oral dose happen after 4 h, and its half-life is roughly 36 h [6]. Erlotinib has a high plasma protein binding of 95%, with the majority of these interactions occurring with albumin and-1 acid glycoprotein [7]. Erlotinib is extensively metabolized by CYP3A4 and, to a lesser extent, by CYP1A2 and the extrahepatic isoform CYP1A1 with metabolites excreted by the biliary system [8]. In numerous investigations [9, 10, 11], high inter-patient variability of pharmacokinetics in erlotinib exposure has been noted.

Erlotinib is classified as class II of the Biopharmaceutical Classification System (BCS), which has low solubility and high permeability [12]. So, using enhanced solubility formulations can increase bioavailability and clinical outcomes [9]. *In vivo* studies are important for these reasons. This study was not performed on the generic form of erlotinib 150 mg tablets that are manufactured by Zistdaru Company and in the Iranian population.

Based on the above discussion, the current single-dose, two-sequence, and randomized crossover trial was designed to compare the bioequivalence of the generic erlotinib formulation developed by Zistdaru Danesh Pharmaceutical Company (Erloxha® 150 mg) as the test and Tarceva® 150 mg as the reference in 24 healthy Iranian volunteers.

MATERIALS AND METHODS

Subjects

The protocol of this study was approved by the Ethics Committee of Tehran University of Medical Sciences, code: IR. TUMS. TIPS. REC.1400.069 and was registered in the Iranian Registry of Clinical Trials (IRCT), IRCT ID: IRCT20200623047902N6. Before enrollment, each volunteer signed a written informed consent form. 24 healthy men were registered as volunteers; their average age was 33.5±8.7 y (range of 21-56 y), their average body weight was 75.2±6.4 kg (range of 65-95 kg), and their average height was 176.3±6.9 cm (ranging from 160 to 190 cm). One month before to the study's start, all of the volunteers had their blood tested for CBC, bilirubin, ALT, AST, FBS, HBS antigen, HCV, HIV, urea, and creatinine. Blood pressure and heart rate were checked before and during the study. Based on the results of the completed clinical assessment, all subjects were found to be healthy.

Drug administration and sample collection

The current single-dose, randomized, two-treatment, two-period crossover study was conducted on healthy Iranian male volunteers. A 1:1 ratio of the reference or test medication was given out at random. Additionally, all subjects fasted for up to 4 h after receiving the medication but at least 10 h before. On the day of the test, a single oral dose of reference and test formulations was administered with 240 ml of water. There was a two-week washout period. Blood samples were taken before the drug administration as well as 1, 1.5, 2, 2.5, 3, 3.5, 4, 5.5, 6, 7, 8, 12, 24, 36, 48, and 72 h later. Blood samples were obtained

via a catheter inserted into an external vein, and they were then placed in tubes containing the anticoagulant EDTA. The collected blood samples were then immediately centrifuged, and the plasma was separated and frozen at–20 °C until analysis.

Chemical

Acetonitrile Pro HPLC (Merck), Zinc Sulfate (Sigma), Methanol Pro HPLC (Merck), Formic Acid (Merck), Erlotinib Hydrochloride working standard (Parsian Pharmaceutical Co.), Sorafenib Tosylate working standard (Parsian Pharmaceutical Co.)

Sample preparation

490 µl of plasma and 10 µl of sorafenib solution as an internal standard (25 µg/ml) were spiked. The prepared solution was mixed for 2 min and then held without shaking for 10 min. After that, 50 µl Zinc Sulfate 1.16 M and 500 µl methanol were added and mixed for 5 min and then held for 10 min without shaking. Then samples were centrifuged at 15 000 rpm for 10 min at 4 °C and the upper phase was injected into LC-MS/MS.

Chromatographic conditions

Quadrupole mass spectrometer Quattro Micro (Waters-Micromass, UK) equipped with an electrospray source (Z-spray) was applied to conduct mass spectroscopy. Filtered samples were injected in a volume of 20 µl into a Thermo (50×4.6 mm, 5 microns) column at 40 °C and separated by Alliance HT separations module 2795 (Waters, Milford, MA, USA), which consists of a quaternary solvent delivery system, degasser, autosampler, column heater. Chromatographic separation was performed at a flow rate of 0.4 ml/min using an elution buffer containing 70% eluent A (0.1% formic acid) and 30% eluent B (100% acetonitrile).

Mass spectrometry measurements were performed on Mass Lynx software, version 4.1. Samples were introduced to API positive source values as follows: Corona 1 (uA); cone 20 V; extractor, 1 V; RF lens, 1 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas flow rate: 1200 L/h Cone gas (nitrogen, 99.99% purity) flow rate: 200 L/h.

Validation procedure

Based on the Food and Drug Administration guidelines, the analytical method was validated in terms of linearity, range, specificity, accuracy, precision, and carryover [13].

Specificity

The specificity test was shown by comparing chromatograms of blank plasma, plasma spiked with 500ng/ml sorafenib as an internal standard (IS) and 25 ng/ml erlotinib as the lower limit of quantification (LLOQ) [14].

Linearity

The LC-MS/MS equipment was used to create and evaluate the spiking standard solutions of erlotinib (in the range of 6.25-3200 ng/ml) and sorafenib (500 ng/ml) as an internal standard in plasma. The final calibration curves included three replicates per calibration concentration, and linearity was assessed by linear regression. The linearity correlation coefficient (R^2) must be more than 0.98.

Accuracy and precision

The low (50 ng/ml), medium (400 ng/ml), and high (2400 ng/ml) Quality Control (QC) plasma samples were used to assess the assay's precision and accuracy. By examining QC samples in triplicate on separate days and the same day, respectively, the inter-day and intra-day assays were determined (table 1). Except for LLOQ (50 ng/ml), where it should not exceed 20% of the RSD%, the precision determined at each concentration should not exceed 15% of the RSD% [15].

Carryover effect

Carryover was assessed during the erlotinib method validation process by injecting blanks after previously injecting samples with a concentration of 3200 ng/ml on the Upper Level of Quantification (ULOQ). The carryover on the blank should not be more than 20% of LLOQ and 5% for internal standards [16].

Stability

Stability studies were conducted by EMEA recommendations. Erlotinib plasma concentrations at low (50 ng/ml) and high (2400 ng/ml) concentrations were prepared in triplicate and stored frozen at-20 °C until analysis. Two-hour thaw, freeze, and thaw cycles were studied for short-term stability tests. Stability was assessed by placing plasma quality control samples at room temperature on the bench for 2 h after preparation. The samples were frozen for 12 h in each cycle and thawed at room temperature to achieve freeze-thaw stability during two cycles. After each storage period, the concentration of erlotinib was compared to the starting concentration, which was established for samples that were freshly manufactured and processed right away. The mean area of the stability solution should be±15% of its freshly prepared solution [17].

Pharmacokinetic analysis

The non-compartmental model was used to calculate or establish the pharmacokinetic parameters. Further, the calculation was performed by WinNonLin 8.1.0.3530 software to determine the area under the plasma concentration-time curve ($AUC_{0\text{-}72}$) from the measured levels, that is, from time 0 to the time of the last quantifiable level (72 h). Other pharmacokinetic parameters that were used to compute the formulations were the maximum plasma concentration (C_{max}) and the time to peak plasma concentration (T_{max}).

Statistical analysis

An analysis of variance (ANOVA) was used to assess the values of C_{max}, $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$, and T_{max} obtained with the two formulations and distinguished between effects resulting from subjects, times, and treatments. Furthermore, log-transformed values of $AUC_{0\text{-}72}$, $AUC_{0\text{-}inf}$, and C_{max} were used as a base to evaluate the equivalence of the two formulations. The 90% CI of the test/reference mean ratios were determined for C_{max}, $AUC_{0\text{–}72}$, and $AUC_{0\text{-}inf}$. The applicable range of 0.8 to 1.25 can lead to bioequivalence between the two formulations [18]. If the P values were less than 0.05, the differences between the two compared parameters were statistically significant.

RESULTS AND DISCUSSION

Analytical method validation

Specificity

Under the chromatographic conditions described, erlotinib and the IS peaks were well resolved. Endogenous plasma components showed no interfering peaks. In fig. 1, typical chromatograms of blank plasma are shown as compared to spiked samples analyzed for a pharmacokinetic study. The average retention times of erlotinib and sorafenib as IS were 0.95 and 1.02 min, respectively.

Linearity

The calibration curve for the determination of erlotinib in plasma was linear over the range of 6.25–3200 ng/ml (fig. 2). The LLOQ was 6.25 ng/ml. The linearity of this method was statistically confirmed. The correlation coefficients (r^2) for the calibration curves were equal to or better than 0.998. The calibration equation is Y=0.00571185X+0.0199664.

Accuracy and precision

The precision and accuracy of the assay were determined from the low (50 ng/ml), medium (400 ng/ml), and high (2400 ng/ml) Quality Control (QC) plasma samples. The inter-day assay was determined by analyzing QC samples in triplicates and was analyzed on three different days. The intra-day precision was determined for each QC sample in plasma, each in triplicate on one day (table 1). The precision value (RSD %) determined at each concentration wasn't more than 6.4%.

Accuracy was expressed as the mean percentage of analytes that were recovered in the assay. The results of the accuracy test are shown in table 2. As shown, coefficients of variation were less than 10%, which is acceptable for routine measurements of the accuracy of the bioanalytical method.

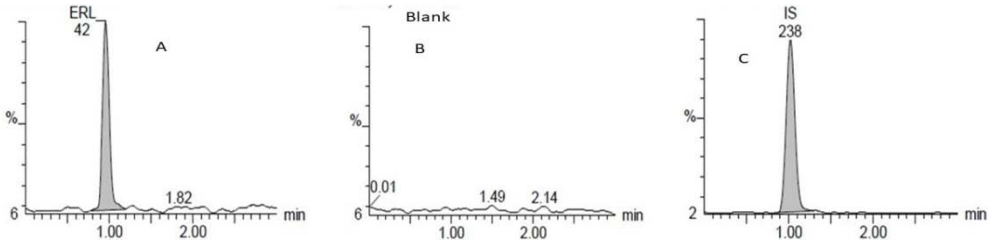

Fig. 1: Chromatograms of (A) blank plasma spiked with 25 ng/ml erlotinib; (B) blank plasma and (C) blank plasma spiked with 500 ng/ml sorafenib as IS

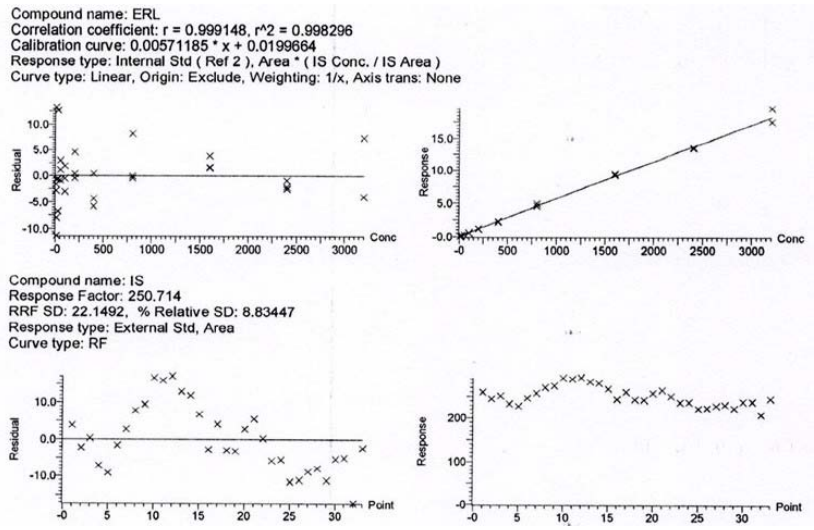

Fig. 2: Calibration curve of erlotinib in plasma

Table 1: Intra-day and inter-day precision of the method for determination of erlotinib in human plasma

	Con. of erlotinib (ng/ml)	Average of drug area/IS area±SD	RSD%
Intra-day precision	50	0.29±0.01	5.0
	400	2.45±0.13	5.3
	2400	13.75±0.29	2.1
Inter-day precision	50	0.31±0.02	6.4
	400	2.31±0.09	3.8
	2400	13.94±0.54	3.9

Note: Data are given in mean±SD, n=3

Table 2: Accuracy of the method for determination of erlotinib in human plasma

	Conc. of erlotinib (ng/ml)	Average of drug area/IS area±SD	RSD%	Deviation
Intra-day Accuracy	50	0.30±0.00	6.2	-2.32
	400	1.64±0.10	5.4	1.64
	2400	13.16±0.60	4.4	-4.14
Inter-day Accuracy	50	0.31±0.01	3.50	0.66
	400	2.43±0.09	3.62	5.42
	2400	14.20±0.40	2.80	3.46

Note: Data are given in mean±SD, n=3

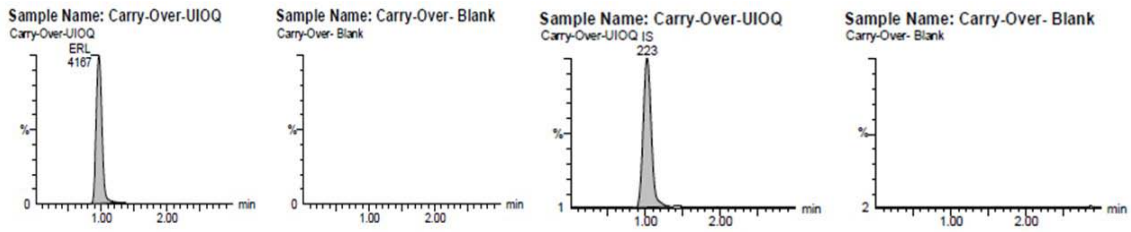

Fig. 3: Carryover effect between high concentration sample of erlotinib and internal standard and blank

Table 4: Stability of erlotinib and IS in the short-term and freeze-thaw cycle

		Area				
		ERL	**IS**	**ERL/IS**	**Con.(ng/ml)**	**%Dev**
Standard	HQC	3984	273	14.60	2554	6.4
		3946	280	14.12	2468	2.8
		4049	289	13.99	2446	1.9
		%Dev				3.7
	LQC	83	288	0.29	47	-6.7
		91	311	0.29	47	-5.1
		93	313	0.30	48	-4.7
		%Dev				5.5
Short Term Stability	HQC	4022	287	14.02	2452	2.2
		4002	305	13.11	2292	-4.5
		3992	293	13.62	2381	-0.8
		%Dev				2.5
	LQC	92	324	0.28	46	-8.7
		96	305	0.32	51	2.1
		91	317	0.29	46	-7.5
		%Dev				6.1
Freeze and thaw stability	HQC	3939	286	13.79	2410	0.4
		3983	295	13.50	2360.4	-1.6
		3910	273	14.31	2502.6	4.3
		%Dev				2.1
	LQC	92	307	0.30	48.5	-3
		89	313	0.29	45.9	-8.3
		96	318	0.30	48.7	-2.5
		%Dev				4.6

As shown in the table above, the difference between the fresh standard and samples that remained at room temperature for two hours and samples that went through two cycles of freezing and thawing is less than 10%.

Carryover effect

Carryover between samples can occur in analytical methods. But in this method, the development carryover effect was evaluated, and no accumulation after a high concentration of erlotinib was seen (fig. 3). So, it could be concluded that there is no need for an essential cleaning procedure between runs.

Stability

The stability of erlotinib and IS in the short term and during freeze-thaw cycles were tested. In all of these stability studies, both erlotinib and IS did not show any significant degradation (table 4). These results confirmed that erlotinib was stable in plasma under storage conditions and during sample preparation.

Pharmacokinetics

24 healthy subjects were randomized and included in the study. The subjects were divided into two groups according to the randomization table. All the volunteers completed the study with no serious adverse effects.

Average plasma concentration-time curves of test and reference products for a single dose of Erlotinib are shown in fig. 4. The reference and test formulations used in the current study have mean AUC_{0-72} values of 12056±3126 ng·h/ml and 12479±2786 ng·h/ml, respectively. The mean C_{max} values for the reference and test formulations were 766±196 ng/ml and 790±146 ng/ml, respectively. Further, the mean T_{max} values were 2.1±0.4 h and 2.1±0.5 h in reference and test formulations, respectively (table 5). The reported values of C_{max} and AUC varied greatly between investigations due to the substantial intra-subject variability [9, 18-22].

The t-test analysis demonstrated no difference between the average values of parameters that resulted from sequencing, period, and administering the test and reference products at the significance level of 0.05. The 90% CIs for the mean ratios of the test versus reference formulation of C_{max}, AUC_{0-72}, and AUC_{0-inf} are equal to 94.61-116.21, 93.39-114.40, and 93.47-117.23, respectively (table 6). Therefore, both are placed in an acceptable range of 0.80 to 1.25 and are found to be bioequivalent.

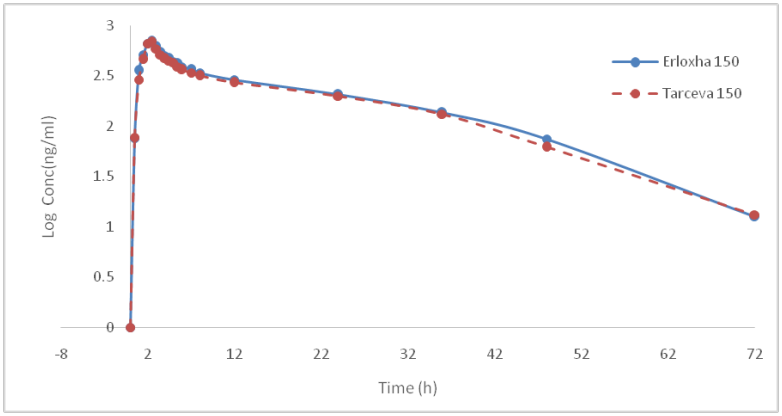

Fig. 4: log-transformed concentration of erlotinib time (h) in healthy volunteers following consumption of Tarceva 150® mg (reference) and Erloxha® 150 mg (test) (n=24). Concentration presented based on mean±SD

Table 5-Summary of pharmacokinetics parameters of Tarceva and Erloxha 150 mg in healthy volunteers

	ID	T_{max} (h)	AUC_{72} (ng. h/ml)	C_{max} (ng/ml)	AUC_{0-Inf} (ng. h/ml)	Kel (1/h)
Tarceva	N	24	24	24	24	24
150 mg	Mean	2.1	12056	766	12571	0.06
	SD	0.4	3126	196	3137	0.02
	SE	0.1	638	40	640	0.0
	CV%	18.7	26	26	25	35.63
	Mean Log	0.7	9	7	9	-2.9
	Geometric Mean	2.1	11649	741	12166	0.1
	Geometric SD	1.2	1	1	1	1.5
	Geometric CV%	19.4	28	27	28	39.3
	CI 95% Lower Mean	1.96	10736	683	11246	0.05
	CI 95% Upper Mean	2.29	13376	848	13896	0.07
	CI 95% Lower GEO Mean	1.93	10374	663	10856	0.05
	CI 95% Upper GEO Mean	2.27	13080	829	13634	0.06
Erloxha	N	24	24	24	24	24
150 mg	Mean	2.08	12479	790	13012	0.06
	SD	0.50	2786	146	2772	0.02
	SE	0.10	569	30	566	0.00
	CV%	24.2	22	19	21	28.37
	Mean Log	0.71	9	7	9	-2.94
	Geometric Mean	2.03	12198	777	12735	0.05
	Geometric SD	1.27	1	1	1	1.34
	Geometric CV%	24.28	22	18	21	29.56
	CI 95% Lower Mean	1.87	11303	728	11842	0.05
	CI 95% Upper Mean	2.30	13656	851	14183	0.06
	CI 95% Lower GEO Mean	1.83	11133	720	11645	0.05
	CI 95% Upper GEO Mean	2.24	13364	839	13927	0.06

Table 6: Bioequivalence results with 90 confidence interval (CI)

Dependent	Ref LSM	Ref LSM_SE	Ref Geo LSM	Test LSM	Test LSM SE	Test geo LSM	Difference	Diff SE	Diff DF	Ref ratio %	Lower CI90	Upper CI90
$Ln(AUC_{inf})$	9.41	0.05	12166	9.45	0.05	12735	0.05	0.07	45.00	104.68	93.47	117.23
$Ln(AUC_{72})$	9.36	0.05	11649	9.41	0.05	12198	0.05	0.07	45.00	104.71	93.39	117.40
$Ln(C_{max})$	6.61	0.04	741	6.66	0.04	777	0.05	0.06	45.00	104.85	94.61	116.21

CONCLUSION

The optimized LC-MS/MS method is selective, accurate, precise, and repeatable. The method is linear over a wide range. The run time is short, and the protein precipitation technique is very simple. It can be concluded that the method is suitable for routine quantification of erlotinib in human plasma.

Overall, there was no significant difference in the pharmacokinetic parameters of the test and reference products when examined *in vivo*. Accordingly, it was concluded that Erloxha® 150 manufactured by Zistdaru Danesh Company, is bioequivalent to Tarceva® 150 manufactured by Roche Company, and both products were similar in terms of the rate and extent of absorption. So, because pharmaceutical equivalence and bioequivalence were concluded, the test product is therapeutically equivalent to the reference product, and the product is pharmaceutically equivalent and interchangeable.

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AUTHORS CONTRIBUTIONS

All authors contributed to the practical work and writing of the manuscript. Sadrai and Ghasemian planned the study, analyzed the data, and prepared the manuscript. Shokri collected blood samples, and Ilka helped Shokri analyze the samples. All authors wrote and revised the manuscript.

CONFLICT OF INTERESTS

The authors have declared that they have no conflict of interest.

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