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The effect of low- and high-dose levothyroxine on the expression, protein level, and function of P-glycoprotein in mice

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ABSTRACT: The study was investigated the effect of different doses of levothyroxine on the mRNA expression, protein level and function of P-glycoprotein. Mice were divided into 6 groups as control, low dose levothyroxine, high dose levothyroxine, fexofenadine, low dose levothyroxine+fexofenadine and high dose levothyroxine+fexofenadine. Mice received levothyroxine at doses of 8 and 80 µg/kg daily for 21 days. Fexofenadine was administered at dose of 40 mg/kg at the 24 h following the last administration of levothyroxine. The mRNA levels and protein level of P-glycoprotein in liver and small intestine were determined by RT-PCR and western blot analysis, respectively. Plasma concentrations of fexofenadine were determined using HPLC. Levothyroxine at low and high doses caused an insignificant increase intestinal mRNA expression of *mdr1a*, while high dose levothyroxine+fexofenadine caused a significant increase. Levothyroxine caused a dose-dependent decrease in intestinal mRNA expression of *mdr1b*. In liver, levothyroxine caused a dose-dependent increase in the mRNA expression of *mdr1a*. Fexofenadine significantly reduced the effect of levothyroxine on mRNA expression of *mdr1a* in liver. Levothyroxine increased the protein level of P-glycoprotein in liver and decrease in intestines. Low dose levothyroxine significantly increased the plasma concentration of fexofenadine. The effects of levothyroxine on the mRNA expression of *mdr1a* and *b* in small intestine and liver and protein level of P-glycoprotein varied depending on the dose, tissue type, and fexofenadine administration.

Keywords: P-glycoprotein, levothyroxine, mRNA, protein level, fexofenadine.

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INTRODUCTION

Thyroid hormones regulate the functions of all organs such as the brain and heart, as well as metabolism, and growth and differentiation in all cells. Thyroid hormones act on thyroid receptors, which are found in the cell nucleus and mitochondria and are a member of the nuclear receptors. Thyroid hormones modulate metabolism with its direct actions on gene expression and by interacting with other nuclear receptors such as peroxisome proliferator-activated receptor and liver X receptor (Mullur et al., 2014). Levothyroxine known as L-thyroxine is used in the treatment of hypothyroidism and is a synthetic form of thyroxine. Levothyroxine, which acts on the thyroid receptors like thyroid hormones, is used in a wide dose range (12.5-300 µg) depending on the degree of hypothyroidism. The thyroid diseases (hypothyroidism and hyperthyroidism) cause changes in the pharmacokinetics and pharmacodynamics of drugs (Burk et al., 2010; O'Connor and Feely, 1987).

Nuclear receptors, also defined as transcription factors regulate the transcription of genes that control a wide variety of physiological events including proliferation, development, metabolism, and homeostasis (Chawla et al., 2001). These receptors also mediate the effects of endogenous substances (steroid and thyroid hormones, vitamins A and D) and various xenobiotics (Markov and Laudet, 2011). In addition, it is stated that nuclear receptors may be effective in the regulation of expression and function of permeability glycoprotein (P-gp) (Fernandez et al., 2004).

P-gp also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 is an important protein of the cell membrane that acts efflux pump (Ambudkar et al., 1999). While P-gp is encoded by a single gene (MDR1) in humans, it is encoded by two genes, *mdr1a* and *mdr1b* in rodents (Hsu et al., 1989). The aminoacid sequences of *mdr1a* and *mdr1b* are highly similar, but their distribution in tissues is different (Brady et al., 2002).

P-gp, which is synthesized in various organs and tissues including liver, intestine, kidney, brain capillary endothelium, is encoded by the MDR1 gene and has a broad substrate specificity (Gül et al., 2016; Thiebaut et al., 1987). These proteins alter the pharmacokinetics and hence the pharmacodynamics of drugs through their effect on the absorption, distribution, and excretion of drugs (Faber et al., 2003; Ho and Kim, 2005). The function of P-gp can be modulated to protect tissues and to alter the effect of drugs. Various

drugs and foods lead to drug-drug and drug-food interactions by causing induction and inhibition of these carrier proteins (Dresser et al., 2003; Greiner et al., 1999; Hebert et al., 1999; Kirn et al., 1999; Matheny et al., 2001; Tras et al., 2017; Westphal et al., 2000).

Fexofenadine, a H₁-receptor antagonist and widely used in treatment, is a substrate of P-gp and CYP3A4. It is largely excreted via feces (80%) and urine (12%) as an unchanged drug (American Society of Hospital Pharmacists, 2010). The modulations of CYP3A and P-gp cause changes in the pharmacokinetics of fexofenadine (Simons and Simons, 1999; Simpson and Jarvis, 2000). The biological half-life and the time to reach peak concentration (T_{max}) of fexofenadine for oral administration were 1.7 and 0.25 hours in mice, respectively, while it was 6.1 and 0.5 hours in rats (Jin and Han, 2010; Medwid et al., 2019).

In the planning of the project, it has been taken into consideration that thyroid hormones have a biphasic effect on proteins and that they increase protein synthesis at low doses and degrade them at high doses (Cicatiello et al., 2018). In addition, the effect of levothyroxine on P-gp mRNA expression *in vitro* was founded to be concentration-related (Mitin et al., 2004), but there is no *in vivo* study about different doses of levothyroxine on the expression and function of P-gp. Levothyroxine may have a significant impact on the occurrence of drug interactions and drug efficacy because the enzymes, receptors, and transporters (e.g., P-gp), which mediate the regulation of the pharmacokinetics and pharmacodynamics of drugs, are composed of proteins. This study aimed to determine the effect of levothyroxine on the expression, protein level and function of P-gp and whether its these effects are dose-related.

MATERIALS AND METHODS

Animals and Study Design

Swiss Albino healthy male mice (total 48, 8-12 weeks, 30 ± 1.92 g) were used in the study. The study was performed in agreement with ethical guidelines and policies approved by the Selcuk University Experimental Medicine Application and Research Center Ethics Committee (SUDAM, 2018-23), which is in harmony with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). During the experimental period, animals were housed in polysulfone cages in a central facility under controlled conditions (12 h light/dark cycle, room temperature of 24 ± 1 °C, and 60% atmospheric hu-

midity) at SUDAM and allowed water and food ad libitum. In the study, the mice were divided into 6 groups consisting of 8 mice. Commercial formulations of levothyroxine (Levotiron, 25 µg tablet, Abdi Ibrahim Pharmaceuticals, Turkiye) and fexofenadine (Fexadyne, 180 mg tablet, Ali Raif Pharmaceutical Industry Co., Turkiye) were used for animal administrations. The doses of levothyroxine and fexofenadine were determined by considering their the LD₅₀ values (Fexofenadine, 2010; Drugbank, 2022) and the previous studies (Cvetkovic et al., 1999; Engels et al., 2016; Kim and Lee, 2019; Cetin et al., 2021). Fexofenadine (1 mg/mL) was dissolved in the distilled water before administration. A 0.5 mL this solution was administered to mice twice with 10 minutes interval. Levothyroxine (2.5 and 5 µg/mL) was dissolved in the distilled water before administration. Mice were administered 0.1 mL of a 2.5 µg/mL solution of levothyroxine for a dosage 8 µg/kg. On the other hand, animals were administered 0.48-0.52 mL of 5 µg/mL levothyroxine solution for a dosage 80 µg/kg. Groups and drugs administered are presented in the Table 1. The period of administration of levothyroxine to mice was determined considering that the half-life of levothyroxine is shorter in experimental animals (1.7-24 h) than 5-9 days in humans (Capen, 1997; Jin and Han, 2010; Medwid et al., 2019).

In the study, fexofenadine was chosen as probe drug of P-gp (Jin and Han, 2010). Blood samples were taken into heparin-containing tubes from the hearts of all animals under xylazine (10 mg/kg, IP) + ketamine (90 mg/kg, IP) anesthesia at 1 h after the administration of fexofenadine and sacrificed via cervical dislocation. The blood samples were centrifuged at 3,000 g for 10 minutes at 4 °C. The plasma samples obtained were stored at -80 °C until the time of analysis. The liver and the upper part of the small intestine (duodenum) of mice were removed following sacrifi-

cation. Tissues were frozen in liquid nitrogen as soon as they were received and stored at -80 °C until RNA isolation.

Laboratory Tests

Isolation of RNA, cDNA Synthesis, and RT-PCR

Total RNA was isolated from tissue samples with Purezole (Biorad, USA) in accordance with the prospectus. Nanodrop (Roche, Swiss) was used to determine the quality and quantity of total RNA obtained. RNAs were analyzed by agarose gel electrophoresis and cDNA synthesis for all samples was performed using the iScript™ cDNA Synthesis Kit (Biorad, USA, Cat. No: 170-8891). RT-qPCR was performed on RT-qPCR Detection System (Biorad CFX Connect Real-Time PCR Detection System, USA), using 4 µL cDNA in a system containing 0.1 µL forward and reverse primer (100 µM), 10 µL SYBR Green Master Mix (SsoAdvanced Universal SYBR Green Supermix Biorad, USA), in a final volume of 20 µL. Cycling conditions were as follows: Pre-incubation for 3 min at 98 °C, followed by 39 cycles of 15 s at 95 °C, 30 s at annealing temperature (Table 2), and 30 s at 72 °C. At the end of RT-qPCR, melting curve was formed by increasing the temperature from 60 °C to 95 °C. One negative control was used in each test. The *mdr1a* and *mdr1b* gene expression was normalized by the β-actin (ACTB) housekeeping gene. Measurements for all samples were carried out in triplicate.

Western Blot Analysis

The liver and small intestine tissues were homogenized using a homogenizer (Isolab, Germany) following addition Xtractor buffer (20x, Takara Bio USA, Inc.) and 10 µL/mL of protease inhibitor cocktail (ProteoGuard EDTA-Free, Takara Bio USA, Inc.). Then, samples were centrifuged at 4 °C, 1,000 g for 10 min. The protein concentration of the obtained

Table 1. The substances administered and their doses in groups^a (n=8)

Group	Levothyroxine	Fexofenadine
C ^b	-	-
F	-	40 mg/kg
LL	8 µg/kg	-
HL	80 µg/kg	-
LLF	8 µg/kg	40 mg/kg
HLF	80 µg/kg	40 mg/kg

^aMice received levothyroxine by daily gavage for 21 days. Fexofenadine was administered by gavage at the 24 h following the last levothyroxine administration; ^bDistilled water was administered orally to the control group.

C, control; F, fexofenadine; LL, low-dose levothyroxine; HL, high-dose levothyroxine; LLF, low-dose levothyroxine+fexofenadine; HLF, high-dose levothyroxine+fexofenadine

Table 2. Sequence of primers, amplicon sizes and their annealing temperatures

Target	Primers	Annealing temperatures	Lenght (bp)*	Accession number
MDR1A-F	5'GGATGAAATTGATAATTTAGACATG3'	54.3 °C	232	NM_011076.3
MDR1A-R	5'TCCATTTATTATGGCACAGAATATA3'			
MDR1B-F	5'AACACAGCCAACCTTGGAAC3'	54.9 °C	180	NM_008830.2
MDR1B-R	5'TGTTGCAATCTTTCCAGCAG3'			
ACTB-F	5'GGCTGGCCGGGACCTGACAGACTAC3'	57.8 °C	150	NM_007393.5
ACTB-R	5'GCAGTGGCCATCTCCTGCTCGAAGTC3'			

*PCR product size (base pair).

supernatant was determined using the BCA Protein Assay Kit (Takara Bio Inc.). Supernatant was stored at -20 °C until analysis. Samples (40 µg) were diluted with an equal volume of 2 x Laemmli buffer, kept at 95 °C for 5 min and centrifuged at 16,000 g for 1 min. Proteins were separated using electrophoresis in an 8% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, and subsequently transferred to the PVDF membrane using the Trans-Blot Turbo Transfer System. The membrane was blocked for 2 hours with 5% skimmed milk powder prepared in tris-NaCl buffer (TBST) containing 0.1% tween-20. Subsequently, the membrane was incubated at 22 °C for 3 hours with P-gp antibodies (1:700, P-gp rabbit polyclonal antibody) prepared in TBST containing 5% skimmed milk powder and washed 5 times with TBST. The blotted membrane was incubated with goat-anti-rabbit secondary antibody (1:4000) conjugated with HRP (horseradish peroxidase) for 1 hour at 22 °C and washed 5 times with TBST. The membrane was incubated for 5 min with ECL substrate containing HRP substrates (Clarity Western ECL substrate, Bio-Rad Laboratories, Inc.) and the relative densities of bands were determined using the ChemiDoc MP system.

Drug Analysis

Fexofenadine Analysis

The plasma concentration of fexofenadine was determined by HPLC-UV system (Shimadzu, Tokyo, Japan) using the method reported by Helmy and El Bedaiwy (2016). A total of 1.5 mL of ether was added to 100 µL of the plasma sample and vortexed for 60 seconds. Subsequently, the ether phase was taken by centrifuging at 3,000 g for 10 minutes, evaporated in a water bath at 50 °C, dissolved with 100 µL of the mobile phase and 25 µL was injected into the HPLC-UV system. Sodium dihydrogen phosphate buffer (20 mmol, pH: 3.0, adjusted with orthophosphoric acid)

in water and acetonitrile (30:70, v/v) were used as the mobile phase. The flow rate of pump was set at 1.0 mL/min. Gemini C18 column (250x4.6 mm; internal diameter, 5 µm; Phenomenex, Torrance, CA) and SPD-10A UV-VIS detector, which set to 215 nm, were used for fexofenadine detection. The column temperature and autosampler were kept at 30 °C and 23 °C, respectively.

The stock solution (100 µg/mL) of fexofenadine hydrochloride prepared in methanol was diluted to prepare the calibration standards (20-2000 ng/mL) and the quality control samples (40, 400 and 2000 ng/mL). The calibration standards prepared using blank mouse plasma in the concentration range of 20-2000 ng/mL were linear with r² of >0.9993. The lowest limit of quantitation (LLOQ) was 20 ng/mL with acceptable validity values (correlation coefficient; <20%, bias; ±15%). The quality control samples were used to determine the recovery and precision of the method. The recovery of fexofenadine from mouse plasma was >89%. The coefficient of intra-day and between-day variation determined for the precision of the method was <8.7%, and the bias for reality was ±8.3%.

Statistical Analyses

The RT-qPCR results performed with primers whose primary efficiency was between 1.9-2.2 were normalized using the 2^{-ΔCt} method according to Livak and Schmittgen (2001) criteria. After normalization, the Anderson-Darling normality test was performed, and it was found that the values showed normal distribution. Analyzes were carried out with the SPSS 22.0 package program. Differences between groups were evaluated using the Tukey test. Graphics were drawn using mean ± SEM values.

The plasma concentrations of fexofenadine were presented as mean ± SD and inter-group differences

were determined using the Mann-Whitney U test. $P < 0.05$ was considered statistically significant in the results.

RESULTS

The results on the effects of levothyroxine on the expression, protein level, and function of P-gp in the liver and intestinal are presented in Figures 1, 2, 3, and 4, respectively. Compared to the control group, levothyroxine at low and high doses caused an insignificant increase in the intestinal mRNA expression of *mdr1a*, while the addition of fexofenadine to the high dose treatment of levothyroxine caused a significant increase ($P < 0.05$, Fig. 1A). On the other hand, levothyroxine caused a dose-dependent decrease in the intestinal mRNA expression of *mdr1b* ($P < 0.05$,

Fig. 1B). In the liver, levothyroxine caused a dose-dependent increase in the mRNA expression of *mdr1a* ($P < 0.05$, Fig. 2A) and a non-dose-dependent increase ($P < 0.05$) in the mRNA expression of *mdr1b* compared to the control group (Fig. 2B). The addition of fexofenadine to levothyroxine significantly caused a decrease in the effect of levothyroxine on the mRNA expression of *mdr1a* in the liver ($P < 0.05$, Fig. 2A).

Consistent with the mRNA levels of P-gp, it was determined that levothyroxine increased the protein level of P-gp in the liver and decreased in the intestines (Fig. 3). While levothyroxine significantly led to an increase in the plasma concentration of fexofenadine at a low dose ($P < 0.05$), it did not cause any change at a high dose ($P > 0.05$, Fig. 4).

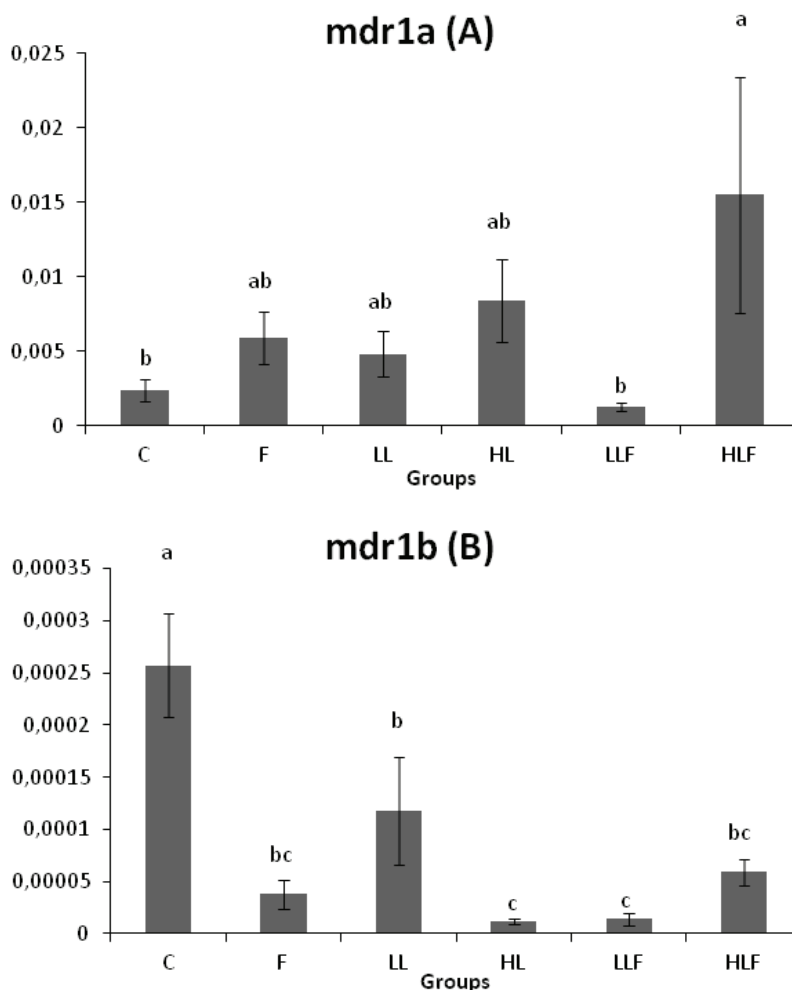


Fig. 1. *mdr1a* (A) and *mdr1b* (B) mRNA levels in the small intestine of mice treated with different doses of levothyroxine ($n = 8$, mean \pm SEM).

a, b, c: Different letters between groups are statistically significant ($P < 0.05$).

C, control; F, fexofenadine; LL, low-dose levothyroxine; HL, high-dose levothyroxine, LLF, low-dose levothyroxine+fexofenadine; HLF, high-dose levothyroxine+fexofenadine

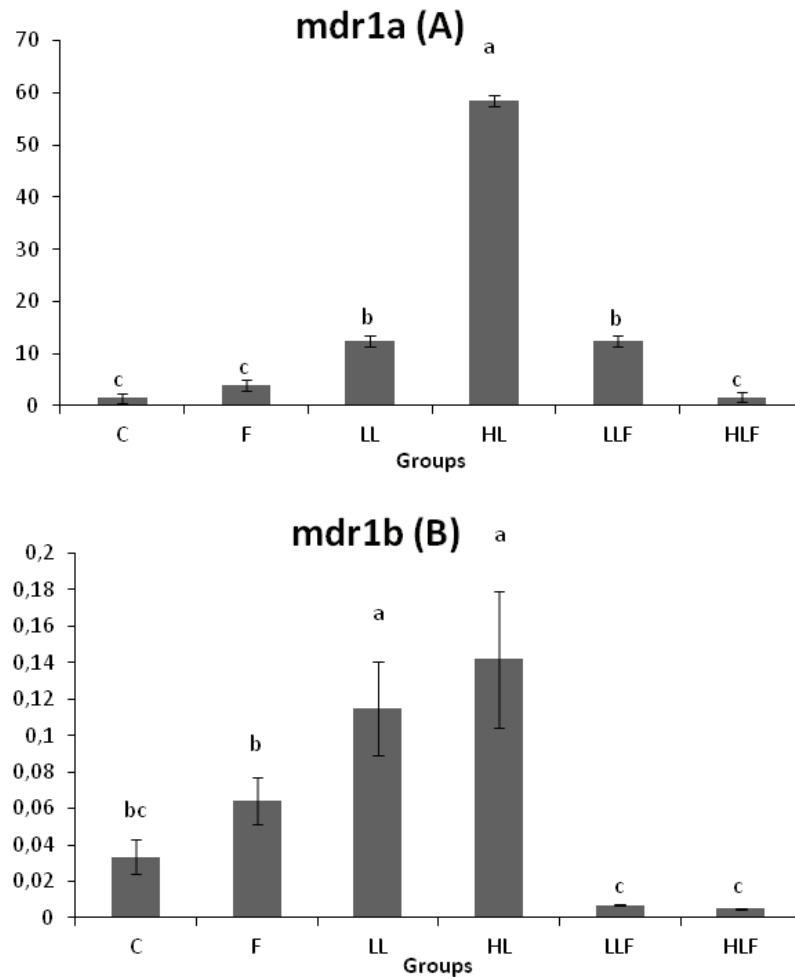


Fig. 2. mdr1a (A) and mdr1b (B) mRNA levels in the liver of mice treated with different doses of levothyroxine (n = 8, mean \pm SEM).

a, b, c: Different letters between groups are statistically significant (P<0.05).

C, control; F, fexofenadine; LL, low-dose levothyroxine; HL, high-dose levothyroxine; LLF, low-dose levothyroxine+fexofenadine; HLF, high-dose levothyroxine+fexofenadine

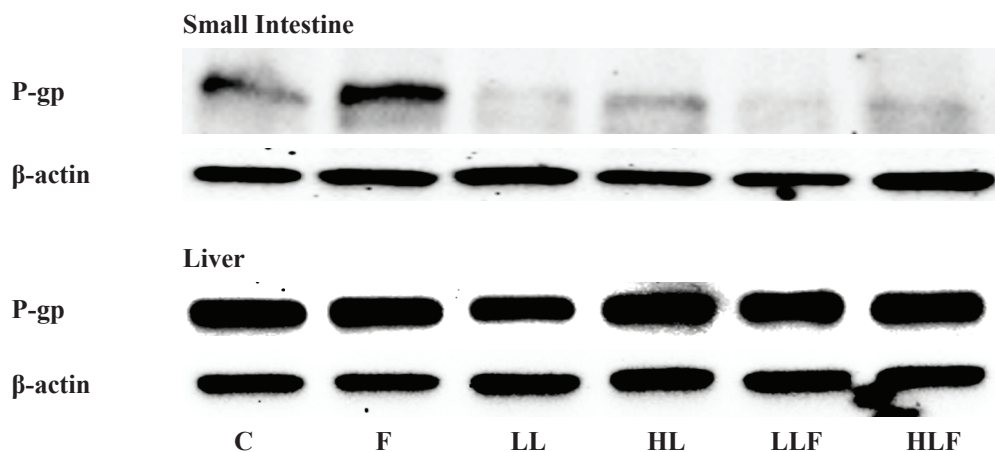


Fig. 3. Western blot analysis of P-gp in liver and small intestine tissues of mice treated with different doses of levothyroxine.

C, control; F, fexofenadine; LL, low-dose levothyroxine; HL, high-dose levothyroxine; LLF, low-dose levothyroxine+fexofenadine; HLF, high-dose levothyroxine+fexofenadine

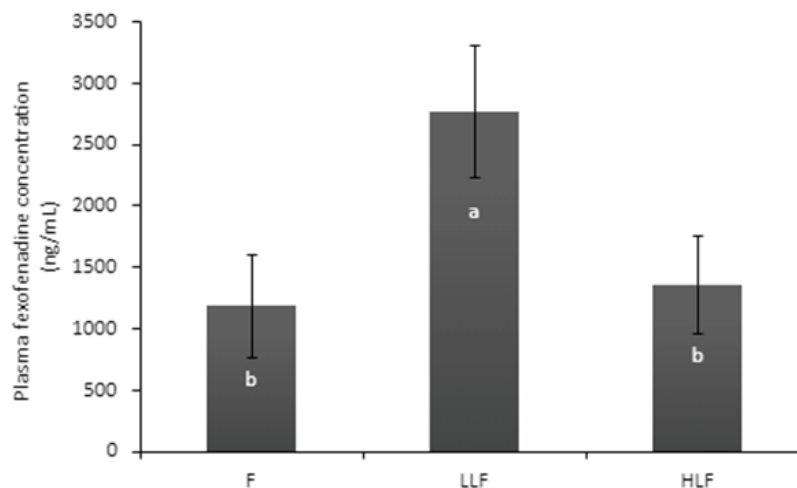


Fig. 4. Plasma concentrations of fexofenadine in rats treated with different doses of levothyroxine (n = 8, mean \pm SD).

a, b: Different letters between groups are statistically significant ($P < 0.05$).

F, fexofenadine; LLF, low-dose levothyroxine+fexofenadine; HLF, high-dose levothyroxine+fexofenadine.

DISCUSSION

P-gp, a transmembrane transporter, plays an important role in the drug disposition in the body and drug-drug / drug-food interaction. This transporter is widely found in organs and tissues that determine disposition of drugs. A lot of studies have been conducted on the induction and inhibition of this most well-characterized protein.

We determined that levothyroxine caused a decrease in the mRNA expression of *mdr1* in the intestines and an increase in the liver depending on the dose. Previous studies have reported that the effects of various drugs and chemicals on P-gp expression were dose and tissue-dependent (Drescher et al., 2003; González-Lobato et al., 2010; Narang et al., 2008; Tanaka et al., 2004; Tras et al., 2021; Zong and Pollack, 2003). In a human study, it has been found that 17-day levothyroxine administration (200 μ g/day) caused a small increase in the mRNA expression of *mdr1* in the upper duodenal region and a significant increase in immunoreactive P-gp (Siegmund et al., 2002). The aforementioned study has similar and different results with our findings. We found an insignificant increase in the mRNA expression of *mdr1a* and a significant decrease in the expression of *mdr1b* in the intestine, however a decrease in the P-gp protein level. The discrepancy between the mentioned study and our findings may be related to differences in used species, tissue, P-gp analysis method, and the dose of levothyroxine. While the upper part of the duodenum for mRNA expression was used in the other study, its entire was used in our study. The distribution of

P-gp in the intestines differs regionally (Mouly and Paine, 2003). The different responses of *mdr1a* and *mdr1b* to drugs may be related to their tissue distribution because in mice *mdr1a* is highest in gastrointestinal tract, *mdr1b* in the ovary and placenta (Cui et al., 2009).

At the stage of determining the effect of levothyroxine on the P-gp function of the study, we determined that low-dose levothyroxine significantly increased the plasma level of fexofenadine compared to the fexofenadine group, while high-dose levothyroxine did not cause any significant change. The reason for the increase in the plasma concentration of fexofenadine caused by low-dose levothyroxine may be related to the decrease in the P-gp level in the intestine. It may also be related to the fact that a low dose fexofenadine causes a slight increase in liver P-gp level compared to a high dose. The slight increase in the P-gp level in the liver may cause a decrease in the excretion of fexofenadine through the liver and thus more passage into the systemic circulation. In addition, the effects of levothyroxine on the P-gp of other organs such as the kidney may have contributed to the difference in the concentration of fexofenadine. It has been reported that 17 days of levothyroxine administration to humans did not cause a change in the pharmacokinetics of talinolol, a specific P-gp substrate, for oral and IV administration (Siegmund et al., 2002).

Previous studies on the interaction of fexofenadine with membrane transporters have reported that transporters such as organic-anion-transporting polypep-

tides (OATP) as well as P-gp are effective on the disposition of fexofenadine in the body (Cvetkovic et al., 1999; Dresser et al., 2002; Jin and Han, 2010; Medwid et al., 2019; Molimard et al., 2004). It is stated that fexofenadine is a substrate for OATP in addition to P-gp, P-gp inhibitors also inhibit OATP and these two carriers are found together in organs such as the liver (Cvetkovic et al., 1999). Medwid et al. (2019) reported that the maximum concentration (C_{max}) and area under curve (AUC) parameters of fexofenadine were 70 and 41% lower, respectively, for oral administration in transgenic mice compared to wild mice, but similar for IV administration. In the same study, the authors determined that the simultaneous oral administration of fexofenadine to wild mice with grapefruit and apple juices caused a decrease in C_{max} (80-88%) and AUC (35-70%). Dresser et al. (2002) reported that grapefruit, apple, and orange juices caused a 70 and 60% reduction in the C_{max} and AUC of fexofenadine in humans, and this reduction was associated with OATP inhibition (Cvetkovic et al., 1999). On the other hand, Jin and Han (2010) have reported that piperine caused an increase 2-fold in the AUC of fexofenadine, and this increase was associated with the inhibition of P-gp in the intestine and liver. The also investigators emphasized that the increase in AUC did not relate to OATP inhibition, because OATP inhibition may have caused a decrease in the AUC. Previous studies indicated that fexofenadine is the substrate of P-gp and OATP transporters, which have an opposite effect on drug behavior (Cvetkovic et al., 1999; Dresser et al., 2002). Therefore, it is understood that it would not be correct to explain the change in plasma concentration of fexofenadine only by the effect of levothyroxine on the P-gp function. In oral administration of drugs such as fexofenadine which is both P-gp and OATP substrate, and poor-metabolized, the degree of inhibition of P-gp can be more important than OATP on the plasma drug concentration.

We determined that the administration of fexofenadine alone caused an insignificant increase in the mRNA level of *mdr1a* in the duodenum compared to the control group. On the other hand, the administration of fexofenadine plus high-dose levothyroxine caused a significant increase in the mRNA level of *mdr1a* but not an increase in the P-gp protein level. The increase associated with fexofenadine in the mRNA level of *mdr1a* may be related to the fact that the presence of the substrate stimulates P-gp expression (Dickenson et al., 2012). In addition, it is indicated that the increase in mRNA may not be reflected in protein synthesis (Shirasaka et al., 2009).

In conclusion, it can be stated that the effect of levothyroxine used for a long-time treatment on P-gp varies depending on the dose and tissue. To determine the effect of inducers such as levothyroxine on the pharmacokinetics of drugs that are substrates for more than one transporter such as fexofenadine, it is necessary to define the effectiveness of inducers on different transporters in many tissues. In addition, it can be pointed out that the metabolism ratios of drugs such as fexofenadine that is a substrate of both P-gp and OATP transporter may play an important role in their interactions with substances (drug/food) that can modulate these transporters.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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