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ORIGINAL ARTICLE

Determination of temporal changes in hepatic drug-oxidizing capacity using plasma metabolite/caffeine ratios in non-pregnant and pregnant goats

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Abstract

Caffeine (CF) is a metabolic probe drug used in the determination of the hepatic drug-oxidizing capacity. The aim of this study was to investigate temporal changes in the hepatic drug-oxidizing capacity using plasma metabolite/CF ratios in non-pregnant goats ($n = 11$) and pregnant goats ($n = 23$). CF (5 mg/kg, intravenous) was administered in six periods (Period 1–6) with 45 days between two periods. The plasma levels of CF and its metabolites, theophylline (TP), theobromine (TB) and paraxanthine (PX), were determined by HPLC-UV. To evaluate hepatic drug-oxidizing capacity in terms of enzymes that play a role in CF metabolism, the plasma metabolic ratios including TB/CF, PX/CF, TP/CF and TB + PX + TP/CF were determined at 10 h following CF administration. Plasma metabolite/CF ratios were similar between non-pregnant and pregnant goats. However, plasma metabolite/CF ratios in Period 3 (45 days in pregnant goats) were significantly higher than those other periods in both pregnant and non-pregnant goats. The effect of pregnancy may not be observed on drugs that are substrates of enzymes involved in CF metabolism in goats.

KEYWORDS
caffeine, goats, hepatic drug-oxidizing capacity, pregnancy

1 | INTRODUCTION

Drug-metabolizing enzymes that mediate drug absorption, tissue distribution and elimination in concert with transporters, are commonly essential for therapeutic drug response. Cytochrome P450 (CYP), which is the dominant enzyme system in drug metabolism, plays an important role in the biotransformation of many drugs. These enzymes expressed in the intestinal, hepatic, and renal epithelium govern the pharmacokinetics of drugs by limiting drug concentrations or making them more concentrated. Therefore, it is important to understand the role of changes in the activities of biotransformation enzymes as determinants of drug concentrations in the domain to achieve better therapeutic results (Kirchheiner & Seeringer, 2007; Rizk et al., 2017; van den Anker et al., 2018).

During pregnancy, many physiological, biochemical, and hormonal changes occur in the body, and these changes cause differences in vehicle protein activity, plasma protein amount and body/water ratio, as well as enzyme activities. In addition, the extent to which these changes occur can be affected by the stage of pregnancy (Jeong, 2010; Morgan et al., 2018). It has been reported that pregnancy in human alters the function of drug-metabolizing enzymes, which affects drug pharmacokinetics (Isoherranen & Thummel, 2013). However, in the veterinary field, nothing is known about the impact of pregnancy on the function of drug-metabolizing enzymes, although there are in vitro and in vivo studies about determining the effect of breed on enzyme activities (Szotáková et al., 2004; Uney et al., 2011). Determining the activity of drug-metabolizing enzymes can provide new insights into potential

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changes in drug pharmacokinetics during pregnancy and for making decisions regarding dosage selection during pregnancy.

The in vivo status of drug-metabolizing enzymes is evaluated by various genotyping and phenotyping methods. However, since genotyping methods are only relevant to the extent to which these enzymes predict the phenotype clinically, it is recommended to use the phenotyping method for the identification of real-time enzyme activity. Consequently, the most appropriate method of identifying true enzyme activity would be phenotyping with a carefully selected probe drug that provides the most clinically suitable knowledge (Faber et al., 2005; Streetman et al., 2000). An ideal probe drug should have low toxicity, low binding to plasma proteins, rapid and complete absorption after oral administration, not being affected by liver blood flow, a significant dependence of its metabolism on the liver, and easy availability.

Caffeine (CF), 1,3,7-trimethylxanthine, is a central nervous system (CNS) stimulant of the methylxanthine class (Carrillo & Benitez, 2000). CF is primarily metabolized to theobromine (TB), paraxanthine (PX), and theophylline (TP) by N-1, N-3 and N-7 demethylation, respectively, in mammals (Kalow & Tang, 1993). N-1 and N7 demethylation of CF were actualized by flavin-containing monooxygenases (Chung & Cha, 1997), CYP1A2 and CYP2E1 while CYP1A2 plays role in N-3 demethylation of CF (Tassaneeyakul et al., 1994, 1992). There are numerous studies and systematic reviews focused on the pharmacokinetics and pharmacological use of CF, effects of CF use on animal health and behavior. It is the most preferred probe drug in determining the hepatic drug-oxidizing (metabolic) capacity because it has many features and criteria that an ideal probe drug should carry (Kalow & Tang, 1993). Studies on the determination of the hepatic drug-oxidizing capacity using CF have been reported in sheep (Uney & Traş, 2011), goat (Uney et al., 2011; Uney & Traş, 2011) and cattle (Danielson & Golsteyn, 1996). However, no information has been found on temporal changes in pregnant goats. Determining the efficiency of drug-metabolizing enzymes during pregnancy can help give information about how pregnancy affects serum concentrations of drugs metabolized by enzymes involved in CF metabolism. In addition, this study may provide basic information about temporal changes in the hepatic drug-oxidizing capacity of pregnant and non-pregnant goats. Therefore, we investigated temporal changes in the hepatic drug-oxidizing capacity using plasma metabolite/CF ratios at 10 h following CF (single dose of 5 mg/kg, intravenous) administration in non-pregnant and pregnant goats.

2 | MATERIAL METHODS

2.1 | Animals

All procedures on goats were approved by the Ethics Committee of the Faculty of Veterinary Medicine, University of Selcuk, Konya, Turkey. Thirty-four Saanen goats (24–28 months) were used in study. The health status of animals were determined by general physical and parasite examination and biochemical and hematological analysis. The animals did not receive any medications up to 2 months prior to the beginning of the study. The animals were randomly divided into two groups (non-pregnant groups as control [$n=11$] and pregnant [$n=23$]). They were placed into two pens of 50 m^2 and were then numbered using oil paint. During the study, non-pregnant and pregnant groups were fed with commercial feed twice daily and alfalfa hay, grass hay, and water were given ad libitum.

2.2 | Study design

In the study, it was ensured that the animals in the pregnant group ($n=23$) became pregnant by natural insemination following estrus synchronization with PRID application. Animals selected for the pregnant group were intravaginally administered progesterone-containing sponges (Chronogest® CR sponge, Intervet) to each goat for 11 days to synchronize the sexual cycles on day 35 after the first CF administration (Period 1). The same animals were administered intramuscularly with PGF2α (125 μg, Estrumate®, Intervet) for two days prior to the day of sponge removal. On the 11th day of the application, CF was applied for the second time (Period 2) to the goats with sponge application. Pregnant goats were identified by transrectal ultrasound performed 19–20 days after naturally occurring mating. CF was administered to the goats determined to be pregnant ($n=19$) at 45 (Period 3), 90 (Period 4), and 135 days (Period 5) of pregnancy and 30 days after birth (Period 6). Four goats that did not conceive were excluded from the study along with the initial CF data samples collected from them. The commercial formulation of CF (Kafedif, 250 mg/mL, injection solution, CEVA) was used for drug administration. During the study, CF (5 mg/kg, IV, single dose) was administered six times at intervals of 45 days to all goats in the non-pregnant and pregnant groups. A previous study found that a single blood sample taken at 10 h after CF administration would be sufficient to assess hepatic drug-oxidizing capacity in goats (Uney et al., 2011). Blood samples were collected into tubes containing EDTA by jugular venipuncture at 10 h after each CF administration. After the blood samples were centrifuged at 4000*g* for 10 min, the plasma obtained was stored at −80°C until analysis. Sample analyses were performed within 1 month following the collection of samples in all periods.

2.3 | Analytical procedure

Plasma were separated and stored at −80°C until analysis, and the concentrations of CF and its metabolites TP, TB and paraxanthine (PX) were determined using high-performance liquid chromatography (HPLC) by a previously described method (Uney et al., 2011). Briefly, 25 μL of β-hydroxyethyl-TP (10 μg/mL, βHE-TP, Sigma) as an internal standard and 750 μL of acetonitrile were added to a test tube containing 250 μL of plasma. After vortexing for 30 s, the samples were centrifuged at 19,000*g* for 5 min. The supernatant was

evaporated to dryness at 40°C under a gentle stream of nitrogen gas and reconstituted in 200 μL of the mobile phase. Using a 0.45 μm syringe filter, 15 μL aliquots of the supernatant was injected into the HPLC system. The HPLC system consists of a pump (LC–20AT), an autosampler (SIL 20A), a column oven (CTO–10A) and a degasser (DGU–14A) and an SPD-10A UV detector. Chromatographic separation was performed using a Phenomenex Gemini C-18 column (250×4.60 mm I.D., 5 μm particle size, Phenomenex), which was maintained at 25°C and the detection wavelength of 273 nm for CF and its metabolites. The mobile phase consisted of 30% methanol in 25 mmol/L sodium acetate buffer (pH 4.0) and it was pumped through the system at a flow rate of 1.0 mL/min.

The method was validated in terms of HPLC system selectivity, sensitivity, linearity, recovery, accuracy and precision in spiked plasma samples. Standard stock solutions of PX, TB, TP and CF (Sigma) were prepared in the mobile phase to obtain a concentration of 1 mg/mL and then mixed for a stock standard mixture (100 μg/mL) consisting of PX, TB, TP and CF. The stock standard mixture (100 μg/mL) was diluted with the mobile phase to prepare working standard solutions at concentrations of 0.01–10 μg/mL. Calibration standards were prepared using working standard solutions in concentrations of 0.01–2 μg/mL for PX, TB and TP and in concentrations of 0.02–10 μg/mL for CF. Quality control samples for PX, TB and TP were prepared in blank goat plasma at low (0.02 μg/mL), medium (0.2 μg/mL) and high (1 μg/mL) concentrations. Quality control samples for CF were prepared at the concentration of 0.04, 0.4 and 4 μg/mL. The selectivity of the method was investigated by extracting blank plasma samples. No interference was observed with biological compounds in plasma. For CF and its metabolites, the calibration standards assayed in triplicate on six different days provided excellent linearity with the correlation coefficient (r^2) > 0.9992. The lowest limit of quantification for CF and its metabolites was 0.02 and 0.01 μg/mL, respectively, with <20% precision and ±15% accuracy. The quality control samples prepared with low, medium, and high concentrations of CF and its metabolites were analyzed in six replicates within 6 days to determine recovery, precision, and accuracy. The recovery was >91% for CF and 93% for PX, TB, TP. Intra- and inter-day precision and accuracy were determined by percent coefficient variation and percent bias, respectively. For CF and its metabolites, the intra-day variability gave good results with precision ranging from 1.52% to 6.62% for CV (coefficient of variation) and accuracy from −3.93% to 5.06% for bias. The inter-day variability was low with precision from 2.08% to 6.51% for CV and accuracy from −4.02% to 5.34% for bias.

2.4 | Data analysis

In the evaluation of the hepatic drug-oxidizing capacity in terms of enzymes that play a role in CF metabolism, the ratio of each metabolite to CF (TB/CF, PX/CF, TP/CF) and ratio of all metabolites to CF (TB + PX + TP/CF) were calculated in blood samples taken at 10 h after CF administration. The CF metabolic ratios to TB, PX and TP were determined by the ratio of each plasma concentration of the metabolites at 10 h to their plasma concentrations sum [(metabolite/TB + PX + TP)$_{10\,h}$] based on the sum of all periods for each group.

All values are shown as the mean ± SD. The normality of the data distribution was assessed using the Shapiro–Wilk test and the homogeneity of variance using the Levene's test. Statistical differences between the metabolite/CF ratios determined in each period in the pregnant and non-pregnant animals were evaluated using the Independent t-test (SPSS 16.0, SPSS for Windows, SPSS Inc.). One-way analysis of variance followed by the Tukey test was used to determine the temporal differences within the group. A value of $p < .05$ was taken for the statistical significance limit.

3 | RESULTS

No clinical finding of adverse drug reaction such as behavior, feed, water consumption, and fecal production was found in pregnant and non-pregnant goats following CF administration. Plasma concentrations of TB, PX and TB and CF at 10 h following the administration of CF in different periods in non-pregnant and pregnant goats are presented in Figures 1–4, respectively. Plasma concentrations of TB, PX and TB at 10 h showed that the demethylation of CF to TB, PX and TP was not constant in non-pregnant and pregnant goats but exhibited temporal changes. It was determined that the CF metabolic ratios of CF to TB, PX and TP based on the sum of all periods for each group were 27/20/53%, respectively, in the non-pregnant group and 28/20/52%, respectively, in the pregnant group. TP was found to be the primary circulating metabolite of CF at 10 h after administration in Saanen goats.

The plasma metabolite/CF ratios calculated at 10 h to determine the hepatic drug-oxidizing capacity in non-pregnant and pregnant goats are presented in Table 1. The plasma metabolite/CF ratios in pregnant goats were not different compared with non-pregnant goats in each period (P > 0.05). However, when evaluated within the group, plasma metabolite/CF ratios in Period 3 (non-pregnant and 45 days of pregnancy in pregnant goats) was found to be higher than those other periods in both groups ($p < .05$).

4 | DISCUSSION

The metabolism of CF is dependent on hepatic biotransformation. Therefore, CF as a probe drug is widely used to evaluate the genetic, environmental, race/species and disease on the activity of particularly CYP1A2 in humans and animals (Kot & Daniel, 2008; Uney et al., 2011; Zanger & Schwab, 2013). CF is transformed to TB and TF by flavin-containing monooxygenases, CYP1A2 and CYP2E1 and to PX by CYP1A2 in rats and humans (Chung & Cha, 1997; Tassaneeyakul et al., 1992; Tassaneeyakul et al., 1994). For these reasons, the hepatic drug-oxidizing (metabolic) capacity for flavin-containing monooxygenases, CYP1A2 and CYP2E1 may be evaluated using the plasma metabolite/CF ratios. In previous studies on sheep and hair goats, it was determined that CF was metabolized to

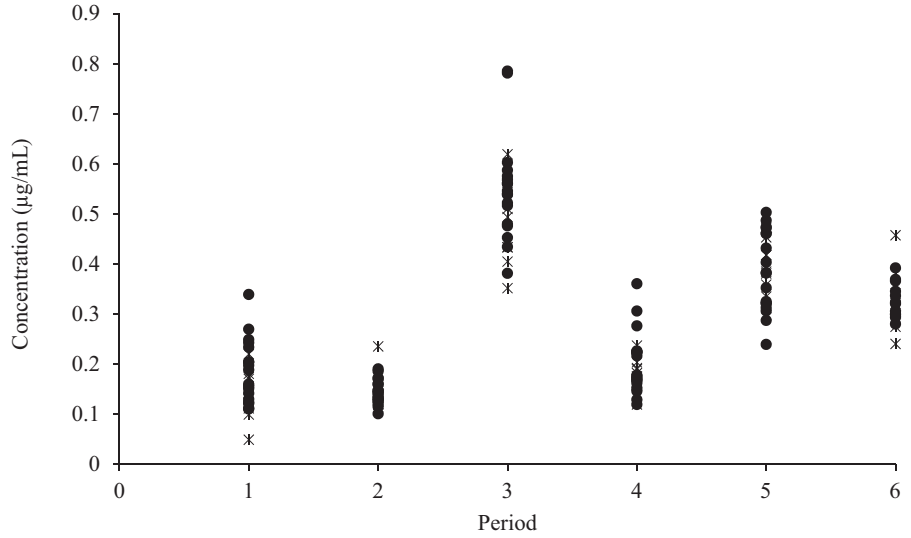


FIGURE 1 Plasma concentrations of theobromine at 10 h following the administration of caffeine at different periods in pregnant and non-pregnant goats. Period 1; first caffeine administration (35 days before PRID (Chronogest® CR sponge) application for synchronization), Period 2; 45 days following the first caffeine administration (last day of PRID application in the pregnant group), Period 3; 90 days following the first caffeine administration (45 days of pregnancy in the pregnant group), Period 4; 135 days following the first caffeine administration (90 days of pregnancy in the pregnant group), Period 5; 180 days following the first caffeine administration (135 days of pregnancy in the pregnant group), Period 6; 225 days following the first caffeine administration (30 day after birth in the pregnant group). o; Pregnant, Ж; Non-pregnant.

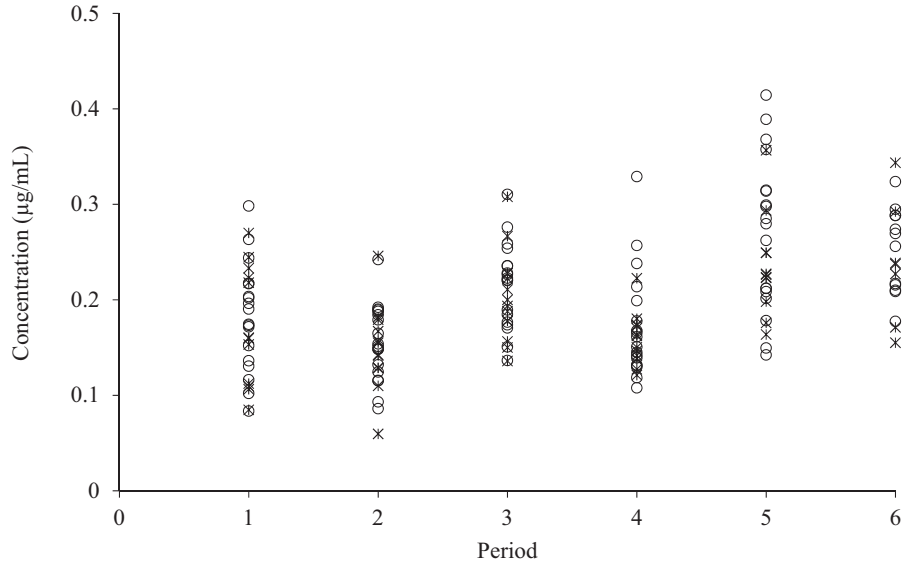


FIGURE 2 Plasma concentrations of paraxanthine at 10 h following the administration of caffeine at different periods in pregnant and non-pregnant goats. Period 1; first caffeine administration (35 days before PRID (Chronogest® CR sponge) application for synchronization), Period 2; 45 days following the first caffeine administration (last day of PRID application in the pregnant group), Period 3; 90 days following the first caffeine administration (45 days of pregnancy in the pregnant group), Period 4; 135 days following the first caffeine administration (90 days of pregnancy in the pregnant group), Period 5; 180 days following the first caffeine administration (135 days of pregnancy in the pregnant group), Period 6; 225 days following the first caffeine administration (30 day after birth in the pregnant group). o; Pregnant, Ж; Non-pregnant.

TB, PX and TP (Uney et al., 2011; Uney & Traş, 2011). Also, previous studies have shown that the primary metabolite of CF is TP in sheep (Danielson & Golsteyn, 1996; Uney & Traş, 2011), PX and TP in hairy goats (Uney et al., 2011), and PX in humans (Berthou et al., 1992) and cattle (Danielson & Golsteyn, 1996). Similarly, it is thought that the primary metabolite of CF could be TP that TB and PX could be produced at approximately the same rate at 10 h after administration in Saanen goats.

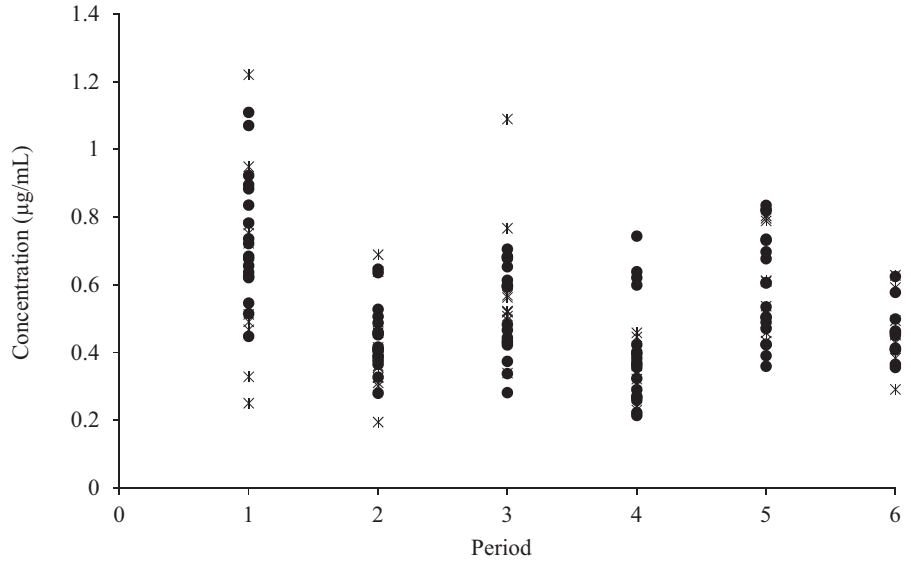


FIGURE 3 Plasma concentrations of theophylline at 10 h following the administration of caffeine at different periods in pregnant and non-pregnant goats. Period 1; first caffeine administration (35 days before PRID (Chronogest® CR sponge) application for synchronization), Period 2; 45 days following the first caffeine administration (last day of PRID application in the pregnant group), Period 3; 90 days following the first caffeine administration (45 days of pregnancy in the pregnant group), Period 4; 135 days following the first caffeine administration (90 days of pregnancy in the pregnant group), Period 5; 180 days following the first caffeine administration (135 days of pregnancy in the pregnant group), Period 6; 225 days following the first caffeine administration (30 day after birth in the pregnant group). o; Pregnant, Ж; Non-pregnant.

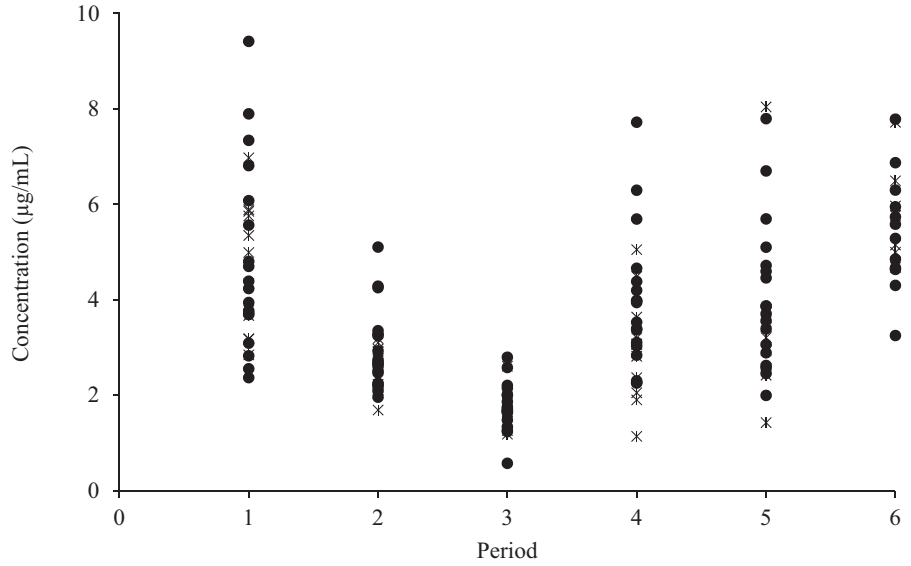


FIGURE 4 Plasma concentrations of caffeine at 10 h following the administration of caffeine at different periods in pregnant and non-pregnant goats. Period 1; first caffeine administration (35 days before PRID (Chronogest® CR sponge) application for synchronization), Period 2; 45 days following the first caffeine administration (last day of PRID application in the pregnant group), Period 3; 90 days following the first caffeine administration (45 days of pregnancy in the pregnant group), Period 4; 135 days following the first caffeine administration (90 days of pregnancy in the pregnant group), Period 5; 180 days following the first caffeine administration (135 days of pregnancy in the pregnant group), Period 6; 225 days following the first caffeine administration (30 day after birth in the pregnant group). o; Pregnant, Ж; Non-pregnant.

Various physiological changes during pregnancy can lead to significant changes in the function of drug-metabolizing enzymes, and this may alter the efficacy or potential toxicities of drugs used during pregnancy (Costantine, 2014; Soldin & Mattison, 2009). The use of pharmacokinetic parameters of CF plays an important role in determining the hepatic drug-oxidation capacity. There are previous studies suggesting a high correlation between CF clearance and metabolite/CF ratios for a single sampling time. (Jodynis-Liebert et al., 2004; Uney et al., 2011). Therefore, some studies suggest calculating a simplified method for determining hepatic

TABLE 1 Plasma metabolite/caffeine ratios calculated at 10 h to determine the hepatic drug-oxidizing capacity in non-pregnant and pregnant goats.

Period	TB/CF	PX/CF			TP/CF		TB + PX + TP/CF	
	p	NP	*p*	NP	*p*	NP	*p*	NP
1	$.04 \pm .02^{c}$	0.03 ± 0.02^{c}	$.04 \pm .01^{c}$	0.04 ± 0.02^{c}	$.17 \pm .07^{b}$	0.17 ± 0.11^{c}	$.26 \pm .09^{b}$	0.24 ± 0.14^{b}
2	$.05 \pm .02^{bc}$	0.06 ± 0.03^{c}	$.06 \pm .02^{bc}$	0.06 ± 0.03^{bc}	$.16 \pm .05^{b}$	0.17 ± 0.09^{c}	$.27 \pm .07^{b}$	0.30 ± 0.15^{b}
3	$.34 \pm 0.13^{a}$	0.29 ± 0.07^{a}	$.13 \pm .05^{a}$	0.12 ± 0.04^{a}	$.33 \pm .10^{a}$	0.35 ± 0.16^{a}	$.80 \pm .26^{a}$	0.77 ± 0.25^{a}
4	$.05 \pm 0.01^{bc}$	0.07 ± 0.04^{c}	$.05 \pm .01^{bc}$	0.06 ± 0.02^{bc}	$.11 \pm .04^{b}$	0.13 ± 0.08^{c}	$.21 \pm .06^{b}$	0.26 ± 0.14^{b}
5	$.10 \pm .04^{b}$	0.14 ± 0.07^{b}	$.07 \pm .02^{b}$	0.09 ± 0.06^{ab}	$.16 \pm .07^{b}$	0.22 ± 0.12^{ab}	$.33 \pm .13^{b}$	0.45 ± 0.25^{b}
6	$.06 \pm .01^{c}$	0.06 ± 0.02^{bc}	$.05 \pm .01^{c}$	0.04 ± 0.02^{c}	$.09 \pm .02^{c}$	0.08 ± 0.03^{c}	$.20 \pm .04^{c}$	0.19 ± 0.07^{c}

Note: ^{a,b,c,d}: Varied characters in the same row are statistically significantly different ($p < .05$). Period 1; first caffeine administration (35 days before PRID (Chronogest® CR sponge) application for synchronization), Period 2; 45 days following the first caffeine administration (last day of PRID application in the pregnant group), Period 3; 90 days following the first caffeine administration (45 days of pregnancy in the pregnant group), Period 4; 135 days following the first caffeine administration (90 days of pregnancy in the pregnant group), Period 5; 180 days following the first caffeine administration (135 days of pregnancy in the pregnant group), Period 6; 225 days following the first caffeine administration (30 day after birth in the pregnant group).

Abbreviations: CF, Caffeine; NP, Non-pregnant group.; P, Pregnant group; PX, Paraxanthine; TB, Theobromine; TP, Theophylline.

drug-oxidation capacity at a single sampling point, using the ratios of CF to all primary metabolites (Tanaka et al., 1992). Therefore, the plasma metabolite/CF ratios were calculated at one time point (10 h) after dosing to assess at which point, it had the highest discriminatory value for the hepatic drug-oxidizing capacity in pregnant and non-pregnant goats.

Our findings showed that the plasma metabolite/CF ratios in pregnant goats were not different compared with non-pregnant in each period. In previous studies in mice and humans, in which CF was used as a probe drug during pregnancy, it was determined that CYP1A2, the enzyme responsible for the primary metabolism of CF, shows a decreasing activity as pregnancy progresses (Koh et al., 2011; Tasnif et al., 2016; Tracy et al., 2005). However, the mechanism of the effect of pregnancy on the regulation of CYPs is still not fully clarified in goats. On the other hand, in a study on rats, an increase in liver size occurred in proportion to the increase in body weight due to pregnancy (Bustamante et al., 2010). Similarly, it was determined that there was a decrease in liver enzyme activity due to the increase in liver size in rats during pregnancy (Symons et al., 1982) (Symons et al., 1982). Even if the enzyme activity decreased in pregnant goats, there may not have been a change in total enzyme capacity due to the increase in the size of the liver due to pregnancy.

In this study, when evaluated within the group, plasma metabolite/CF ratios in Period 3 (45 days of pregnancy in pregnant goats) was found to be higher than those other periods in both non-pregnant and pregnant groups. Intraindividual variability is observed in CYP1A2 enzyme activity, mostly due to environmental factors (Faber et al., 2005). Previous studies in goats have shown that metabolic activities, such as, body condition, milk production, serum chemistry and vitamin status, are affected depending on the season. However, these studies have not been conducted on drug-metabolizing enzymes (Nardone et al., 2010; Nouws et al., 1988; Yokus et al., 2006). In a study conducted on rats, hamsters and fish, environmental temperatures have changed the weight and composition of the liver, causing a change in drug-metabolizing capacity (Dewaide, 1970). In addition, while it was determined that seasonal changes were effective on drug-metabolizing enzymes in frogs, no study in this regard was found in goats (Miura et al., 1986). However, plasma concentrations of CYP3A4 drug substrates in humans have been determined to show seasonal variations, consistent with the stimulatory effect of vitamin D on drug metabolism (Lindh et al., 2011). The data obtained in the third period of the study may show that seasonal factors may cause changes in enzyme activity in pregnant and non-pregnant goats. However, the variability of CYP1A2 due to seasonal factors cannot be clearly explained by the data obtained in this study. Each environmental factor should be evaluated in detail to shed light on the actual variation in enzyme activity due to environmental factors, and genetic analysis at the molecular level should be performed to provide conclusive evidence that CYP1A2 exhibits an environmental genetic polymorphism.

This experimental study has limitations. In this study, hepatic drug oxidation capacity was evaluated with a single blood sample taken 10 h after CF administration in goats. Therefore we cannot be sure whether there are different metabolites in circulation. However, in a study using different time points and pharmacokinetic parameters in goats, due to data showing that a single blood sample taken 10 h after CF administration would be sufficient to determine hepatic drug oxidation capacity in goats (Uney et al., 2011); we hypothesize that the primary metabolite of CF in Sanen goats 10 h after administration is TP and that TB and PX can be produced at approximately the same rate.

This study has some strengths. The number of animals used in the study increased the reliability of the results and offered us the opportunity to examine the temporal changes in the hepatic drug-oxidizing capacity of goats during gestation. No undesirable effects of CF administration on behavior, feed, water consumption and fecal production were observed in pregnant and non-pregnant goats.

5 | CONCLUSIONS

Since changes in enzyme capacity due to pregnancy-induced physiological changes in goats cannot be fully revealed by phenotyping, more research is needed to clarify the mechanisms underlying the genetic polymorphism in CYP1A2 and pregnancy-related differences in goats.

AUTHOR CONTRIBUTIONS

FA: writing – original draft, project administration, review and editing; SUC and AMK: study design and experimental applications; OC: analytical procedure and data analysis; KU original draft and review & editing.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine (University of Selcuk, Konya, Turkey), under protocol number 2015/62.

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