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Original Research

Effects of Bedding Substrates on Microsomal Enzymes in Rabbit Liver

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Previous studies have reported that housing rats and mice on softwood beddings induce microsomal enzymes. To date, no published studies investigate effects of softwood beddings on microsomal induction in rabbits. The purpose of this study was to determine whether microsomal enzymes, primarily cytochromes P450 3A and 2B, were induced in rabbits exposed to commonly used bedding substrates. Rabbits were placed in cages 7.6 cm above 1 sheet of 24 × 36 in. postconsumer recycled paper, approximately 16 cups (130 ounces) of pine shavings, or no substrate. Positive-control rabbits were given either rifampin (50 mg/kg) or phenobarbital (60 mg/kg) intraperitoneally once daily for 5 d. At 2, 7, and 14 d after placement in test cages, rabbits were euthanized and the livers harvested. Microsomal pellets were prepared from the livers and used in an erythromycin N-demethylase assay (to determine CYP3A activity) and a pentoxyresorufin-O-deethylation assay (to determine CYP2B activity). Although the levels of enzyme induction varied slightly in both assays, statistical significance was not reached compared to the positive-control levels. These results indicate that neither CYP450 3A or 2B enzymes are induced by exposure of rabbits to pine shavings or paper substrate as noncontact bedding for up to 14 d.

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; CYP450, cytochrome P450

Microsomal enzymes are found throughout the body and are responsible for metabolizing a variety of drugs, endogenous steroid hormones and prostaglandins, dietary products, and other substances. The liver contains the majority of the microsomal enzymes, which can be induced or inhibited by numerous factors.[7] In research, unexpected induction or inhibition of these enzymes has the potential to confound data, particularly regarding drug metabolism and duration of action.[2,5,9-12] For example, in the mid-1960s, it was found that rats previously housed on corncob bedding had shortened hexobarbital and pentobarbital sleeptimes when changed to softwood bedding.[8] In other studies, rodent microsomal enzymes were induced by pine bedding, resulting in more rapid metabolism of pentobarbital and shortened effect of the drug.[2,5,10,11]

In the laboratory setting, bedding serves several purposes. For rabbits, bedding is most often placed below the cage (noncontact) to catch and bind feces and urine for easy removal and to provide odor control. Common types of bedding include virgin wood pulp, paper, and softwood (such as pine) shavings. Although the types of housing and bedding to which animals are exposed often are not considered during the design of a study, in some situations, the environment of the animal has the potential to influence or confound findings.

Previous studies have shown that pine shavings, a commonly used softwood bedding, can induce microsomal enzymes in the rat.[12] To date, no studies have been done to determine if this induction also occurs in rabbits. It is assumed, although not experimentally tested, that even noncontact exposure to softwood bedding can induce liver microsomal enzymes in rabbits. One method of studying microsomal enzyme expression is to determine the activity of individual cytochrome P450 (CYP450) enzymes. The purpose of this study was to determine the effect of pine and paper used as noncontact bedding substrates below the cage on CYP450 activity (specifically cytochromes P450 3A and 2B) in the livers of rabbits.

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Materials and Methods

Chemicals. Tris HCl, magnesium chloride, potassium chloride, sucrose, Nash reagent (ammonium acetate, acetylacetone, and glacial acetic acid), potassium phosphate, erythromycin, β nicotinamide adenine dinucleotide phosphate (NADPH), trichloroacetic acid, formaldehyde, resorufin, and pentoxyresorufin were purchased from Sigma (St Louis, MO). The Lowry assay was performed using a modified Lowry Protein Assay Kit (Pierce, Rockford, IL).

Animals. This study was approved by the Johns Hopkins University Institutional Animal Care and Use Committee and was in compliance with the Animal Welfare Act and *The Guide for the Care and Use of Laboratory Animals*.[6]

A total of 33 male 14-wk-old SPF New Zealand white rabbits (Robinson Services, Winston-Salem, NC) weighing 5 to 6 kg were used. These rabbits were from a colony that was negative for cilia-associated respiratory bacillus, *Encephalitozoon cuniculi*, *Pasteurella multocida*, and *Treponema cuniculi*, and prior to the experiment, they had no exposure to any substrate other than concrete, which was cleaned of waste material daily. Upon arrival, each group of rabbits (no substrate, paper, pine) was housed in separate rooms to prevent cross-exposure of substrates from different experimental groups. All rabbits were housed in 62 × 42 × 64-cm (length × height × depth) stainless steel cages (Hazleton Systems, Aberdeen, MD) with flooring 7.6 cm above the bedding substrate. Immediately upon arrival, the rabbits were housed above the test substrate for 2, 7, or 14 d, with substrate changed twice weekly. The rooms had 10 to 12 complete air changes per hour with an automatically controlled

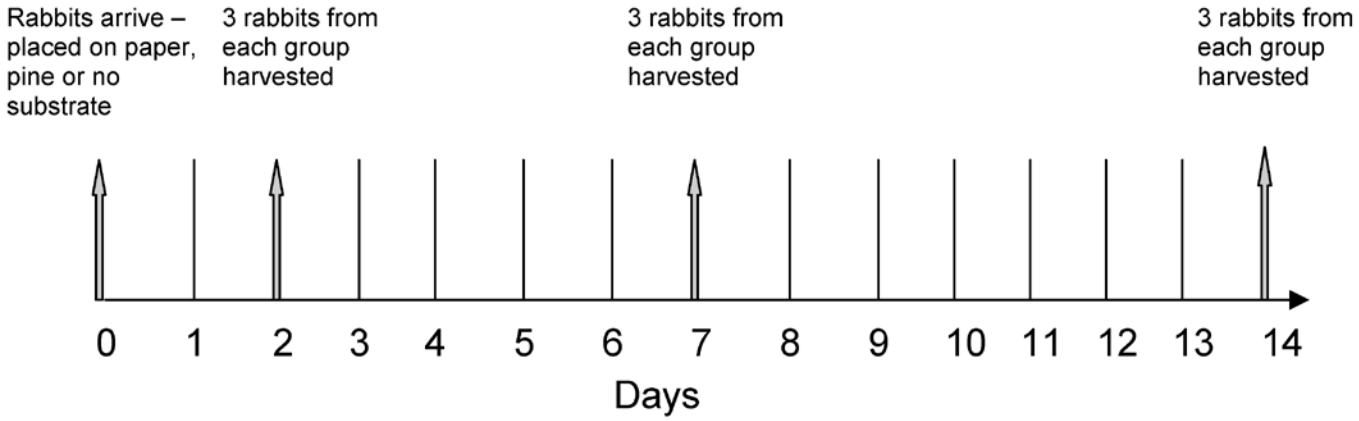

Figure 1. Timeline displaying the types of bedding and termination points. The 3 groups (negative control, paper, and pine shavings) each contained 9 rabbits, which were further divided into 2-, 7-, and 14-d subgroups. Each subgroup contained 3 rabbits.

photoperiod of 12-h light daily. The room temperature was maintained at 21.1 to 22.2 °C and relative humidity at 50% to 55%. The rabbits were fed free choice with commercially available high-fiber pellets (High Fiber Rabbit Diet #2031, Harlan, Frederick, MD) and reverse-osmosis water provided via an automatic watering system. Each rabbit received timothy hay (Johns Hopkins Farm, Baltimore, MD) once weekly.

We randomly allocated 27 of the rabbits into 3 groups. The negative-control group consisted of 9 rabbits housed with no substrate. The remaining 18 rabbits were divided equally into 2 exposure groups: 9 rabbits were exposed to 1 sheet of paper substrate made from 24 × 36-in. 2-ply postconsumer recycled paper (Cage Liner, Acme Paper Products, Savage, MD) folded to fit the cage pan, and 9 were exposed to 16 cups (130 ounces) of kiln-dried pine shaving substrate (Lab Grade Pine Shavings #7088, Harlan). Because 2 different CYP450s were being evaluated, there also were 2 different positive-control groups consisting of 3 rabbits each, all housed without bedding substrate. The CYP450 3A positive-control group was given rifampin (50 mg/kg; Bedford Lab, Bedford, OH) intraperitoneally once daily for 5 d. The CYP450 2B positive-control group was given phenobarbital (60 mg/kg; Abbott, Abbott Park, IL) intraperitoneally once daily for 5 d.

Rabbits were euthanized at 3 time points after heavy sedation with intramuscular ketamine (25 mg/kg) and xylazine (3 mg/kg) followed by pentobarbital (200 mg/kg) by intracardiac injection. After administering the ketamine and xylazine to the first 3 rabbits, we found it difficult to administer the pentobarbital via the ear vein and therefore switched to intracardiac administration. The difficulty in administering the drugs through a peripheral vessel may have been due to hypotension or vasoconstriction. For consistency, all subsequent rabbits were euthanized via intracardiac injection after heavy sedation.

We evaluated 3 time points: 2, 7, and 14 d. Three rabbits from the negative control (no bedding) and the 2 treatment groups (pine or paper) were euthanized at 2 d from the beginning of the exposure. In addition, 3 rabbits from each group were euthanized at 7 d or 14 d. The positive-control treatment rabbits were euthanized 24 h after the last dose of rifampin or phenobarbital was administered. Figure 1 shows the time line of exposure and endpoints.

Immediately after euthanasia, livers were excised and the gall bladder carefully removed. The livers were frozen in liquid nitrogen, freeze-fractured into small pieces, and frozen at –80 °C for later processing to prepare microsomes.

Microsome preparation. Microsomes were prepared as described by Davey[3] by weighing 1 g of frozen liver and homogenizing in 4 ml of Tris–sucrose buffer by use of a glass homogenizer. The tissue homogenates were centrifuged at 10,000 × *g* for 15 min. The supernatant was removed and centrifuged at 45,000 × *g* for 45 min. The resulting microsomal pellet was resuspended in 2 ml of Tris–sucrose buffer, hand-homogenized, divided into 3 equal aliquots, and frozen at –80 °C for later determination of CYP450 activity. The protein concentration of the microsomal fraction was determined by use of a modified Lowry kit (Pierce), with bovine serum albumin as a standard.

Erythromycin N-demethylase assay (CYP3A). CYP450 3A activity was determined by use of the erythromycin N-demethylase assay.[13] Frozen microsomes were thawed on ice, and 20 µl of microsomes were incubated in 680 µl of 50 mM KPO_4 buffer (pH 7.25), 0.1 ml of 150 mM $MgCl_2$, and 0.1 ml of 10 mM erythromycin (a CYP3A substrate). The tubes were incubated for 3 min at 37 °C, and the reaction was initiated with 10 mM NADPH. The reaction was terminated at 10 min with the addition of 12.5% trichloroacetic acid. Each sample was centrifuged at 3000 × *g* for 10 min. One ml of supernatant was added to 1 ml Nash reagent and placed in a 50 °C water bath for 30 min. The samples were allowed to cool and the absorbance read at 412 nm (DU800 spectrophotometer, Beckman Coulter, Fullerton, CA). A formaldehyde curve was used as a standard to determine the erythromycin N-demethylase activity.

7-Pentoxyresorufin-O-deethylation assay (CYP2B). CYP450 2B content was determined by use of the 7-pentoxyresorufin-O-deethylation assay.[4] Frozen microsomes were thawed on ice, and 400 µg protein was incubated in a 0.1 M Tris buffer (pH 7.8) with 0.53 mM pentoxyresorufin, the CYP2B enzyme substrate, in a volume of 200 µL. The reaction was initiated with the addition of 20 mM NADPH to each well and allowed to incubate for 40 min at 37 °C. The production of resorufin was determined by use of a dual-scanning microplate fluorescence spectrometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale CA) set at an excitation wavelength of 510 nm and an emission wavelength of 586 nm. A resorufin standard curve was used to determine the 7-pentoxyresorufin-O-deethylation activity.

Data analysis. Comparisons between means were performed by 1-way analysis of variance and Bonferonni comparison testing (Prism 4, Microsoft, Seattle, WA). Differences between means were considered to be statistically significant when $P < 0.05$. All data are presented as mean ± 1 standard deviation.

Results

The effects of different bedding substrates on the CYP450 3A enzyme activity in rabbit hepatic microsomes at 3 different times of exposure are shown in Figure 2. Of the treatment

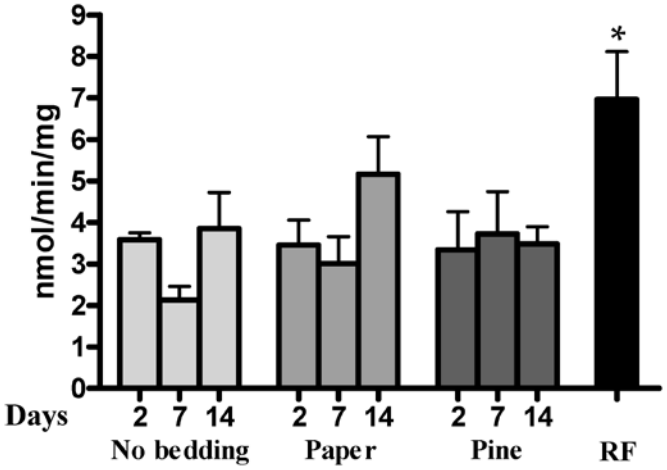

Figure 2. Erythromycin N-demethylase assay (CYP3A) comparing the 3 treatment groups with the rifampin (RF) positive-control group. The bars represent the mean ± 1 standard deviation (n = 3). *, $P < 0.001$ versus RF group.

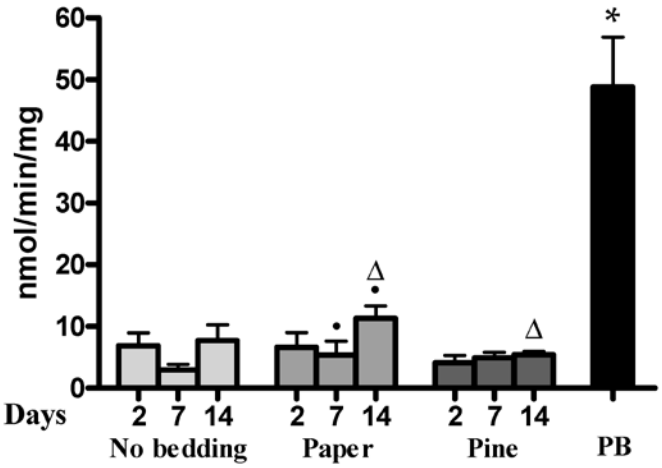

Figure 3. 7-pentoxyresorufin O-deethylation assay (CYP2B) comparing the 3 treatment groups with the phenobarbital (PB) positive-control group. The bars represent the mean ± 1 standard deviation (n = 3). *, $P < 0.001$ versus PB group; •, $P < 0.05$ between groups; Δ, $P < 0.05$ between groups.

groups, rabbits exposed to no substrate for 7 d had the lowest mean activity (2.14 ± 0.22 nmol/min/mg), and those exposed to paper substrate for 14 d had the highest mean activity (4.71 ± 0.43 nmol/min/mg). Comparing the mean enzyme activities among biologically relevant treatment groups, such as all time points within a treatment group (for example, pine), revealed no statistically significant differences. A second analysis was performed to compare the means for a single time point (for example, day 7) across the 3 treatment groups; this comparison also did not reveal any statistically significant differences. However, in the CYP3A assay, all treatment group means were statistically different from the positive-control rifampin-treated group mean activity (6.97 ± 0.81 nmol/min/mg). This finding indicates that there was no CYP3A induction under our experimental treatment conditions and confirms the validity and range of the erythromycin N-demethylase assay.

Figure 3 shows the effects of bedding substrate on the CYP450 2B enzyme activity in rabbit hepatic microsomes at 3 different times of exposure. Of the treatment groups, rabbits exposed to no substrate for 7 d had the lowest mean activity (2.98 ± 0.63 nmol/min/mg), and those exposed to paper substrate for 14 d had the highest mean activity (11.33 ± 1.42 nmol/min/mg). Comparing the mean enzyme activities among biologically relevant treatment groups, such as all time points within a treatment group (for example, pine), revealed only 1 difference that reached statistical significance. The enzyme activity of the 7-d paper group (5.37 ± 1.6 nmol/min/mg) was significantly ($P < 0.05$) less than that of the 14 d paper group (11.33 ± 1.42 nmol/min/mg). A second analysis was performed to compare the means for a single time point (for example, day 7) across the 3 treatment groups. The enzyme activity of the 14-d paper group (11.33 ± 1.42 nmol/min/mg) was significantly ($P < 0.05$) greater than that of the 14-d pine group (5.45 ± 0.38 nmol/min/mg). However, in the CYP2B assay, the activities of all treatment groups differed significantly ($P < 0.05$) from that of the positive-control phenobarbital-treated group (48.84 ± 8.02 nmol/min/mg). This finding indicates that there was limited CYP2B induction and confirms the validity and range of the 7-pentoxyresorufin O-deethylation assay.

Discussion

The primary objective of this study was to determine whether the choice of noncontact bedding substrate for rabbit cages would induce hepatic microsomal enzyme activity, as has been shown in rats and mice.[2,3,5,8-12] To determine whether microsomal enzymes were induced, we evaluated the activities of 2 specific cytochrome P450 enzymes. Cytochrome P450 is a system of phase I enzymes important in biotransformation. The highest concentration of these enzymes is found in the endoplasmic reticulum (microsomes) of the liver.[7] This system primarily is responsible for metabolizing endogenous hormones and prostaglandins and exogenous xenobiotics.[7] Induction of these enzymes can affect the duration of action of numerous drugs.[7] The liver contains many different gene families of cytochrome P450s, which are designated by numbers (1, 2, 3, and so forth) and determined by amino acid sequence analysis. These gene families are further divided into subfamilies, including 1A, 2A, 3A, 2B, 2E, and so forth, with each subfamily then divided into different members (1A1, 3A4, 2E1, and so forth).[7] Xenobiotics, defined as administered drugs or environmental agents, either induce or inhibit certain subfamilies of cytochrome P450.[7] Enzyme assays using specific chemical substrates, such as erythromycin (3A enzymes) or pentoxyresorufin (2B subfamily), have been developed to determine whether induction or inhibition has occurred.[4,13] In studies involving xenobiotics, animals with unexpected induction of their CYP450 system due to environmental agents may have shortened duration of action of drugs (for example, pentobarbital).[2,5,10,11] This situation potentially could lead to administration of a nontherapeutic dose of a drug, thus confounding the treatment protocol. This complication can affect drug safety studies and pharmacokinetic studies as well as have clinical implications.

CYP3A is the most abundant CYP450 enzyme in the body.[7] This large subfamily is important in the biotransformation of numerous drugs including macrolide antibiotics (rifampin, erythromycin), dexamethasone, and phenobarbital.[7] In the current study, we chose the erythromycin N-demethylase assay to determine whether the CYP3A subfamily was induced. Rifampin was given to rabbits and used as the positive control because it is a known inducer of CYP3A.[7] The drug was administered intraperitoneally, on the basis of other protocols.[1]

CYP2B is another subfamily of CYP450 and is well known for its metabolism of barbiturates. We chose the 7-pentoxyresorufin O-deethylation assay to determine whether the CYP2B subfamily was induced. Phenobarbital was used as the positive control

because it is a known inducer of CYP2B.[1] The drug was administered intraperitoneally, on the basis of other protocols.[1]

The rabbits were housed above pine shavings, paper, or no substrate for as long as 2 wk. For consistency among groups, the rabbits were housed with the substrate immediately on arrival to the facility. A period of acclimation was not included because doing so would have made the exposure time of the no-substrate group longer than those of the 2 substrate groups.

For the analysis of the CYP3A and CYP2B enzyme activities, the treatment group means were compared based on biologic relevance. One analysis compared the mean enzyme activity within a treatment group (for example, all means within the paper group were compared). Another analysis compared the mean enzyme activity for a specific time point across the 3 treatment groups (for example, the means of all 3 treatment groups on day 2 were compared). Finally, all treatment group means then were compared with the mean enzyme activity of the positive control for the enzyme studied.

In the CYP3A assay, rifampin was used as a positive control because it is a proven inducer of cytochrome P450 3A[1]. In the comparisons within treatment groups and between time points, statistical significance was not achieved. When the treatment groups were compared with the rifampin group, the rifampin group was significantly different from each treatment group. This finding indicates that CYP3A was not induced in the treatment groups and that induction did occur in the positive-control samples.

In the CYP2B assay, phenobarbital was used as a positive control because it is a proven inducer of cytochrome P450 2B[1]. In the mean comparisons within treatment groups, there was statistical significance when the 7-d paper group was compared with the 14-d paper group. The increase in enzyme activity occurred at 14 d. In the second comparison across the same time point among the 3 treatment groups, statistical significance was achieved when the 14-d paper group was compared with the 14-d pine group. The level of induction was greater in the 14-d paper group. This finding may not be biologically relevant because it was not found among all treatment groups at day 14. Comparison within a treatment group likely is the most biologically relevant comparison, as we observed for the mean comparison of the paper group. Our results indicate that limited CYP2B enzyme induction was induced within the paper group, but not under other biologically relevant experimental conditions.

Lastly, when the treatment group means were compared with the phenobarbital group mean, we found that the phenobarbital group mean was significantly different from that for each treatment group and that the expected enzyme induction did occur under positive-control conditions.

The substrate and time period associated with the lowest activity in both assays occurred in the rabbits exposed to no substrate for 7 d. Rabbits exposed to paper for 7 d also had low enzyme activity. It is difficult to know whether this finding represents a true biological effect. The low level of enzyme activity on day 7 in 2 of the treatment groups may be due to natural fluctuations in enzyme activity or coincidence. One theory is that there may have been an unknown inducer at the vendor site or during shipment that resulted in a residual increase in enzyme activity on day 2 that resolved to baseline activity on day 7. However, this effect was not apparent in the pine substrate group, making the plausibility of this theory flawed.

The 14-d paper group had the highest activity and, although not statistically significant when compared with the positive controls, comparing the 14-d pine group and the 7-d paper group achieved significance. This increased level of enzyme activity in the 14 d paper group was interesting and unexpected. It is unclear why this group had higher activity than the pine group or the 7 d paper group. The differences in the mean enzyme activity may be within the normal variation in the small sample size. Another possibility is associated with the presence of a CYP2B enzyme inducer in the paper. The paper that was used was postconsumer recycled paper. This type of paper may contain various dyes, bleach, and other chemicals that may have affected enzyme activity and may require a 2-wk exposure to result in enzyme induction. There may also be residual chemicals in the paper associated with processing that resulted in the increase in enzyme activity. Further investigation into the effects of postconsumer recycled paper may be warranted.

Previous rodent studies have demonstrated enzyme induction with direct bedding exposure.[2,5,10,11] Because our study did not demonstrate an overall significant induction, we theorized that there were several potential reasons why rabbit liver CYP450 enzymes 3A and 2B were not induced significantly compared with positive-control levels. Rabbits frequently are housed with the substrate below the cage. Because our rabbits did not have skin contact with bedding, we hypothesized that inhalation or ingestion of aromatic compounds from the bedding substrate would induce CYP450 enzymes. In previous rat and mouse studies, the bedding was in direct contact with the animal, and skin, inhalation, and ingestion are likely exposure routes. This difference (lack of skin contact) may play a significant role in the diminished microsomal enzyme induction in rabbits. Another possible factor is that the bedding substrate was 7.6 cm below the cage in our studies. Had the bedding substrate been closer to the rabbits, exposure would have been greater and the results might have been different because the concentration of the aromatic hydrocarbons might have been higher.

In addition, the diminished enzyme induction observed in the rabbits compared with that observed in rat studies may result from reduced exposure to volatilized chemicals due to differences in airflow in the rat cage versus the rabbit cage. Rabbits typically are housed in metal-barred cages, allowing for considerable ventilation. Rodents often are housed in enclosed filtered cages with less overall ventilation compared with that of rabbit cages. Recently, enclosed rodent cages have been connected to ventilated rack systems that have improved the ventilation of the cage markedly. Given housing conventions at the time, older studies on rat exposure to bedding presumably were done in open static rodent cages. This difference in cage ventilation may result in an increase in the concentration of aromatic compound exposure from the bedding in rodents compared with rabbits. In addition, studies done in the 1960s and 1970s most likely used softwood shavings that were not kiln-dried or heat-treated. This treatment has been shown to reduce the levels of aromatic hydrocarbons[9,12] and could also result in decreased exposure. In our study, the shavings were kiln-dried, thus reducing the amount of aromatic compounds.

In conclusion, this study shows that the bedding substrate below the rabbit, unlike for rats and mice, likely will not affect studies addressing drug safety or pharmacokinetics or research involving induction of CYP 3A or 2B. However, further studies should be undertaken to determine whether direct contact or changes in airflow would alter the results we obtained or lead to induction of other CYP450 enzymes.

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