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ASSESSMENT AND DETECTION

The Effect of Hair Pigment on the Incorporation of Fatty Acid Ethyl Esters (FAEE)

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Abstract — Aims: The objective of the current study was to determine whether FAEE incorporation is affected by hair pigmentation. **Methods:** Black hooded LE rats were injected intraperitoneally daily with ethanol. Prior to dosing, black and white patches of fur were shaved and analyzed for baseline levels of FAEE using an adapted extraction procedure and GCMS method. Once the shaved 'patches' had grown back they were re-sampled along with hair outside the 'patches', referred to as 'no patch' hair, and tested for post-treatment FAEE levels in the same manner. Blood was also sampled for pharmacokinetic analysis of ethanol. **Results:** Total FAEE levels were significantly higher in post-treatment hair (black and white) compared to baseline (pre-treatment) levels. Total FAEE levels were also significantly higher in post-treatment 'patch' hair (black and white) compared to 'no patch' hair. No significant differences were found between post-treatment black and white hair. The FAEE profiles were similar between black and white hair, with FAEE levels being highest for ethyl myristate, followed by ethyl stearate, palmitate, and then oleate. **Conclusion:** FAEE incorporation into hair does not appear to be affected by hair pigment, which is in congruence with what is known about the chemistry of drug–melanin interactions. This is important in avoiding potential bias and discrimination in the interpretation of alcohol abuse based on hair color.

INTRODUCTION

In recent decades, hair testing as a method for the detection of drugs of abuse has been gaining popularity because of its unique ability to reliably measure chronic and or past substance use. Lately, there has been an increased need for the development of such a biomarker for alcohol exposure. A prominent example is the case of diagnosing fetal alcohol spectrum disorder (FASD). FASD is a prevalent cause of neurocognitive handicap among children in North America (Abel and Sokol, 1986, 1987). One of the most serious challenges in diagnosing FASD is the need to establish evidence of excessive maternal drinking during pregnancy as a major diagnostic criterion (Chudley *et al*., 2005). However, maternal self-reports of alcohol use are often unreliable (Ernhart *et al*., 1988; Russell *et al*., 1996; Alvik *et al*., 2006; Wurst *et al*., 2008), and therefore a reliable objective biomarker that can detect past chronic alcohol use would prove invaluable in this area.

In 2001, Pragst and colleagues established a biomarker in hair that can reliably assess chronic heavy alcohol use by the measurement of non-oxidative metabolites of ethanol, fatty acid ethyl esters (Pragst *et al*., 2001). Since its introduction, the FAEE hair test has been increasingly used in the medical context of alcohol abuse and the medicolegal context of 'driving under the influence'(Pragst and Yegles, 2008; Wurst *et al*., 2008) because of its high sensitivity and specificity in the detection of excessive drinking in adults (Wurst *et al*., 2004; Pragst and Balikova, 2006). Recent advances in research have also suggested that the FAEE hair test has potential for use as a new diagnostic tool for FASD and FASD research, further expanding its utility (Kulaga *et al*., 2008).

The increase of the FAEE hair test's applications has prompted us to ask whether FAEE incorporation is susceptible to hair color bias, as has been the case with certain other drugs. Several studies have demonstrated that some drugs can accumulate preferentially in pigmented hair (Green and Wilson, 1996; Gygi *et al*., 1996, 1997; Reid *et al*., 1996; Slawson *et al*., 1996; Wilkins *et al*., 1998; Hubbard *et al*., 2000; Kronstrand *et al*., 2003; Scheidweiler *et al*., 2005; Kintz, 2007). The issue of whether or not hair color creates bias in interpreting drug exposure is serious and may discriminate against individuals with dark hair. The interactions of most significance are those that occur between basic/cationic drugs, such as cocaine or methamphetamine, with the anionic centers of the melanin granules (Nakahara *et al*., 1992, 1995; Nakahara and Kikura, 1996; Gygi *et al*., 1997; DeLauder and Kidwell, 2000; Borges *et al*., 2001b, 2003). Neutral drugs have not been found to accumulate substantially in hair or to preferentially accumulate in pigmented hair because of this fact. The objective of the present study was to determine whether FAEE incorporation is affected by hair pigmentation, as the test is becoming more widely used. We hypothesize that FAEE incorporation will not be influenced by hair pigment because of their neutral, lipophilic, nature.

MATERIALS AND METHODS

Animals and treatment protocol

The study protocol was approved by the Animal Care Committee of the Hospital for Sick Children in Toronto. Hooded black and white Long–Evans (LE) rats ($n = 6$) were obtained from Charles River Canada Inc., St. Constant, Quebec, and housed together in groups of three with a 12-h day/night cycle. Beginning on the first experimental day, two 3 × 3 cm 'patches' of fur were shaved from each animal, one black and one white, and tested for baseline levels of FAEE. Prior to dosing, each animal was weighed and then dosed to achieve a peak blood ethanol concentration of 200 mg/dl. Rats were administered 16% w/v ethanol in saline injections by i.p. route according to a protocol developed by Bloom *et al*. (1982) that dictates the volume of injection in order to correct for the inadequacy of g/kg formulations, which do not produce uniform blood ethanol concentrations because they do not take into account the fact

that developmental increases in liver weight and function do not keep pace with developmental increases in body weight.

The rats were dosed daily for 3 weeks (Monday to Friday) when it was determined that a sufficient amount of hair (∼1.00 cm in length) had grown back in the shaved 'patches' to be re-sampled for FAEE analysis. Black and white fur outside of the 'patches' was also sampled for analysis. On the last day of dosing, blood samples were taken at 30, 60, 90, 120, 180 and 240 min post-dose through a catheter implanted in the jugular vein and analyzed for ethanol. The animals were anesthetized with 3% isoflurane for all procedures except weighing.

Blood ethanol analysis

Blood ethanol levels were measured by Headspace Gas Chromatography (Agilent 6890 N GC with Headspace Sampler G1888). Samples of 10 μl of serum were diluted to 100 μl and mixed briefly (3–5 s) on the vortex with 1.0 ml of internal standard (*n*-propanol in water). Samples were then analyzed by the headspace GC-FID against known calibrators and quality control solutions.

Pharmacokinetic analysis

Total systemic exposure to ethanol per dose, area under the blood-versus-ethanol time curve (AUC), Vmax and Km were estimated for ethanol, by fitting plasma concentrations to time using Modkine v.1.2.2 (Biosoft, Cambridge, UK). Initial parameters were estimated with Scientist for Windows v. 3.0 (Micromath, Saint Louis, MO, USA). Michaelis–Menten elimination kinetics was used as the kinetic model, and the maximum enzyme velocity (Vmax; mg/dl/min) and the Michaelis–Menten constant, Km, (Cp at which Vmax is half, mg/dl), were obtained; the fitting procedure was repeated three times. The area under the blood-versus-ethanol time curve ($AUC_{0-t;}$ mg/dl/min) was then estimated by non-compartmental procedures.

Chemicals and reagents

Ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate, dimethylsulfoxide (DMSO), and *n*-heptane were obtained from Sigma Aldrich. Deuterated internal standards of D-5 ethyl myristate, D-5 ethyl palmitate, D-5 ethyl oleate and D-5 ethyl stearate were obtained from the laboratory of Dr Fritz Pragst, Berlin, Germany. Salts for the phosphate buffer, potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate $\times$ $2H_2O$ ($Na_2HPO_4 \times 2H_2O$), were obtained from Fischer Scientific and BDH Laboratory Supplies, respectively.

Hair extraction

A method adapted from the published method, Pragst *et al*., (2001), was used for FAEE extraction and GCMS analysis. Briefly, 20–25 mg of hair was weighed for each sample into 4 ml extraction vials and chopped finely into 1- to 3-mm pieces. A multipoint standard curve with concentrations ranging from 0.0125 ng/mg hair (0.25 ng/sample) to 2.5 ng/mg hair (50 ng/sample) was prepared by spiking naïve rat hair with fresh stock solutions of FAEE standards (ethyl myristate, ethyl palmitate, ethyl oleate, ethyl stearate). Deuterated FAEE (D-5 ethyl myristate, D-5 ethyl palmitate, D-5 ethyl oleate and D-5 ethyl stearate) were used as internal standards for each sample. Extraction solvents (500 μl DMSO and 2 ml heptane) were added to each sample. The samples were then placed in a VWR mini shaker at 1200 rpm for 15 h. The samples were then cooled at 4°C for 30 min to freeze the DMSO phase, and then the heptane phase was decanted into 10 ml solid-phase microextraction (SPME) vials. Heptane was then evaporated at 40°C under nitrogen gas. To the residues, 1 mL of phosphate buffer (0.1 M, pH 7.6) was added, and the vials were capped and put into the AOC 500 Shimadzu autosampler to undergo headspace solid-phase microextraction (HS-SPME).

Table 1. Retention times and *m/z* values of molecular weight and qualifier ions of FAEE and D5-FAEE

Analyte	Retention time (min)	Molecular weight ion	Qualifier ions
D_5-Ethyl myristate	10.35	261	93, 106, 162
Ethyl myristate	10.38	256	88, 101, 157
D_5-Ethyl palmitate	11.38	289	93, 106, 162
Ethyl palmitate	11.40	284	88, 101, 157
D_5-Ethyl oleate	12.22	315	93, 106
Ethyl oleate	12.24	310	88, 101
D_5-Ethyl stearate	12.30	317	93, 106, 162
Ethyl stearate	12.33	312	88, 101, 157

HS-SPME conditions

Samples were preheated for 5 min at 90°C and 250 rpm agitation. Headspace absorption occurred for 30 min at 90°C at 150 rpm agitation, and desorption was for 15 min at 260°C. The agitation mode was 60 s rotation to the right, 30 s rest interval, followed by 60 s rotation to the left, followed by 30 s rest interval, etc.

GC/MS conditions

A Shimadzu GCMS-QP2010 was used for GCMS analysis. For chromatographic separation, a Varian Factor Four, VF-Xms, capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) was chosen. The temperature of the injector, interface, ion source and quadrapole was at 260°C, 310°C, 230°C and 70°C, respectively. The temperature program was as follows: 2 min at 70°C, then 20°C/min up to 300°C, hold 0.5 min at 300°C. Analytes were quantified based on their molecular weight ion. Retention times and *m/z* values of molecular weight and qualifier ions of FAEE and D5-FAEE that were used for quantification and identification of the species are shown in Table 1. Final results are reported as cumulative FAEE (total ethyl myristate, palmitate, oleate and stearate) per mg hair.

Analytical precision

The method was evaluated for its reproducibility prior to sample testing. Intraday coefficient of variation (cv) values were calculated from six replicate analyses of direct injection of three different concentration levels (5, 10 and 20 ng/μl) of pure standard on the same day. Interday cv values were calculated by single injections repeated over three days using the same concentration levels. All intraday and interday cv values were below 3% and 5% for each FAEE, respectively.

Reproducibility was similarly evaluated in extracted white and black rat hair samples. Six replicates at three different concentration levels (2, 20 and 50 ng/ml), for each white and black hair samples, were spiked with FAEE standards, extracted and quantified. Intraday and interday cv values for all esters were below 18% and 16%, respectively.

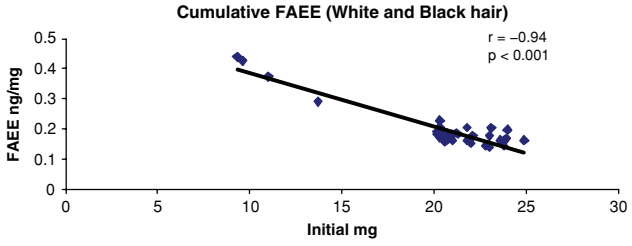

Fig. 1. Initial sample weight (mg) of all black and white hair samples plotted against their cumulative FAEE results per mg hair.

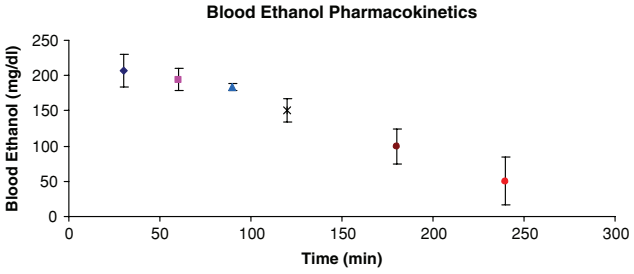

Fig. 2. Blood ethanol concentrations in Long–Evans rats ($n = 5$).

Efficiency of extraction

The efficiency of extraction was estimated by spiking both white and black rat hair with three different concentrations of FAEE (six replicates each), followed by extraction. Analogous concentrations of pure standards were then directly injected by syringe into the GCMS, with six replicates each. Direct area counts were then averaged among the six extracted replicates processed by HS-SPME and compared to the average of six directly injected replicates. Percent extraction efficiency values for the four esters in both white and black hair ranged between 8 and 76%, and 5 and 55%, respectively.

Data analysis

FAEE levels between categories and the effect of initial hair weight on the efficiency of extraction were evaluated using paired *t*-tests and Pearson product moment correlation tests using Sigma Stat software, version 3.1.

Upon analyzing the data, a significant effect of initial hair sample weight on the efficiency of extraction was detected using the Pearson correlation. There was a significant inverse relationship between mg hair used in the extraction and FAEE per mg hair quantified (Fig. 1). After separating the data by white hair, black hair and treatment category (baseline hair, post-treatment 'patch' and post-treatment 'no patch'), the effect of sample weight on extraction efficiency was significant in all categories for black hair ($r = -0.82$, $P = 0.05$; $r = -0.90$, $P = 0.02$; $r = -0.90$, $P = 0.01$, respectively), and in post-treatment 'patch' category for white hair ($r = -0.95$, $P = 0.01$); however, the effect was not significant in baseline or post-treatment 'no patch' categories for white hair ($r = -0.66$, $P = 0.16$; $r = -0.59$, $P = 0.22$). Therefore, white samples were not adjusted for comparison between baseline and post-treatment levels of FAEE, whereas all black samples were standardized to 20 mg to correct for the effect of initial hair weight on the efficiency of extraction. Therefore, for comparison of FAEE levels between black and white hair, all black and white post-treatment 'patch' samples that were standardized to 20 mg were compared.

RESULTS

Ethanol pharmacokinetics and FAEE profile

Ethanol disposition exhibited the Michaelis–Menten kinetics (Fig. 2). The average Vmax, Km and AUC_{0-t} (area-under-the-blood-versus-ethanol-time-curve) values were 0.86 ± 0.19 mg/dl/min, 3.23 ± 1.58 mg/dl and $27{,}897 \pm 5434$ mg/dl/min, respectively ($n = 4$). No significant correlations using the Pearson

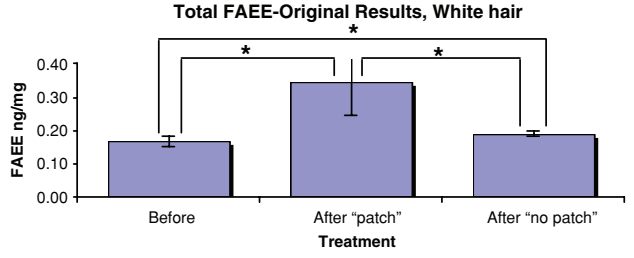

Fig. 3. Mean cumulative FAEE levels in white rat fur, before and after ethanol treatment, not standardized to 20 mg (paired *t*-test comparison). *Indicates statistical significance as measured by the paired *t*-test.

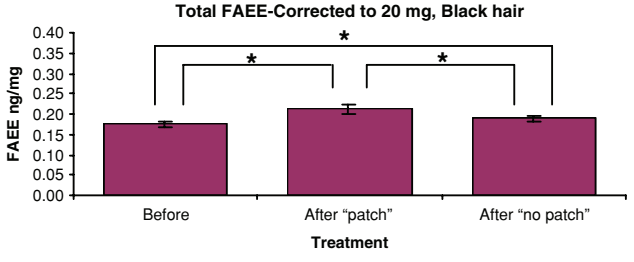

Fig. 4. Mean cumulative FAEE levels in black rat fur, before and after ethanol treatment, standardized to 20 mg (paired *t*-test comparison). *Indicates statistical significance as measured by the paired *t*-test.

test were found between hair FAEE (cumulative FAEE, ethyl oleate or cumulative FAEE corrected to 20 mg) and blood ethanol AUC.

The FAEE profile was similar in both black and white fur, with average levels of FAEE being highest for ethyl myristate, followed by ethyl stearate, palmitate, and then oleate.

Baseline and post-treatment FAEE

Using paired *t*-tests, cumulative FAEE levels were significantly higher in rat hair after ethanol treatment for both black and white hair (Figs 3 and 4). Significantly higher levels of FAEE were found when comparing baseline (pre-treatment) levels to both the "patch" hair that grew back post-treatment, and the surrounding "no patch" hair (the hair that was not shaved). Significantly higher levels of FAEE were also found in the "patch" levels, compared to the surrounding "no patch" treatment hair.

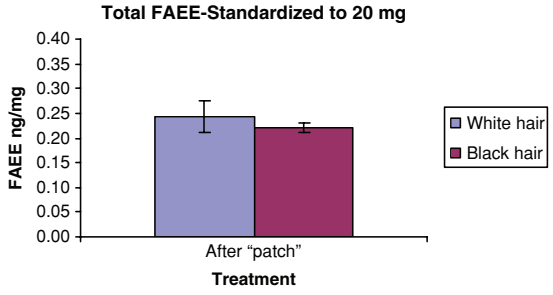

Fig. 5. Mean cumulative FAEE concentrations in white and black hair after standardization to 20 mg (paired *t*-test comparison).

Effect of hair pigment on FAEE

Using the paired *t*-test, no significant difference could be found between FAEE levels in white and black post-treatment 'patch' hair (Fig. 5).

DISCUSSION

The purpose of the present study was to determine whether FAEE incorporation is affected by hair pigmentation, and our results suggest that it is not. The current study did reveal an effect of initial sample weight on the efficiency of extraction, which required standardization of samples to 20 mg for the comparison of FAEE levels between black and white hair. This effect has been noted previously in human hair by Pragst *et al.* (2001) who reported decreasing extraction yields as initial sample amount was increased. Because in the present study we were only able to collect between 10 and 15 mg of hair for some of the post-treatment 'patch' samples in white hair, standardization to 20 mg was of particular importance, but not all samples (baseline, and post-treatment 'no patch' white hair) could be standardized, as the effect size was not statistically significant in those treatment groups. Therefore, only post-treatment 'patch' samples were compared between black and white hair. However, post-treatment 'patch' hair accumulated the highest amount of FAEE, significantly higher than pre-treatment levels and post-treatment 'no-patch' levels, therefore making it the most useful treatment category for comparison.

Comparison of post-treatment 'patch' hair revealed no significant difference in FAEE incorporation between pigmented and non-pigmented hair, supporting our hypothesis that FAEE incorporation is not affected by hair pigmentation. FAEE are neutral, lipophilic molecules, and melanin granules favor interactions with basic/cationic substances; consequently such an interaction between FAEE and melanin was not expected (Nakahara *et al.*, 1995; Nakahara and Kikura, 1996; Kikura and Nakahara, 1998; Borges *et al.*, 2003). Similar results have been reported by colleagues for the hydrophilic molecule, ethyl glucoronide (EtG) (Appenzeller *et al.*, 2007). EtG is a minor metabolite formed during ethanol metabolism when ethanol is glucoronidated with activated glucoronic acid instead of water (Pragst and Yegles, 2007). Its use as a biomarker in hair for the assessment of excessive alcohol use has recently been investigated, and Appenzeller *et al.* (2007) demonstrate no significant difference in the incorporation of EtG between pigmented and non-pigmented hairs of humans with graying hair ($n = 21$). In fact, the investigators report a correlation of 0.99 between EtG results from pigmented and non-pigmented hair. Therefore, it appears that substances can be largely incorporated into hair yet not be influenced by hair pigment, as appears to be the case for FAEE.

The present study is the first to describe the use of the LE rat as a model for hair FAEE research. The LE rat has some differences from the human that need to be acknowledged, although altogether it provides a suitable model for this research. Firstly, LE rat hair contains only eumelanins (the pigment responsible for dark brown or black hair tones) (Slawson *et al.*, 1998), whereas human hair contains both eumelanins and pheomelanins (the pigment responsible for red tone) (Borges *et al.*, 2001a). Pigment granules comprise less than 3% of the total hair fiber mass (Robbins, 2002), and the concentration of eumelanin in LE rat hair has been measured to be 17.56 ± 0.61 μg/mg in pigmented hair (undetectable in non-pigmented hair) (Slawson *et al.*, 1998), and to range from 2 to 15 μg/mg in human hair of varying colors from individuals of different ethnicity (Borges *et al.*, 2001a). Different colors of human hair contain different proportions of eumelanin and pheomelanin, with black hair containing ~99% eumelanin and 1% pheomelanin, brown and blond hair containing ~95% eumelanin and 5% pheomelanin, and red hair containing ~67% eumelanin and 33% pheomelanin (Borges *et al.*, 2001a). However, the fact that LE rat hair may contain a higher concentration of eumelanin than human hair, and that it does not contain pheomelanin, does not affect the primary end point of the present study, to determine if FAEE incorporation is affected by hair pigment. Studies have shown that although some drugs interact with pheomelanins, those interactions are typically much weaker than those with eumelanins, and consequently of less importance (Gygi *et al.*, 1996; Potsch *et al.*, 1997; Slawson *et al.*, 1998; Mars and Larsson, 1999; Rollins *et al.*, 2003; Borges *et al.*, 2003). Furthermore, if FAEE were interacting with eumelanin during incorporation leading to increased incorporation in pigmented hair, the fact that LE rats have a greater concentration of this pigment present in their hair relative to humans would result in a pronounced effect of higher FAEE levels in pigmented hair using the LE model. However, this was not observed in the present study confirming the hypothesis that FAEE incorporation is not affected by hair pigment.

Another difference between the LE rat and humans is that the metabolic rate of the LE rat is much higher, with Vmax values of 0.86 ± 0.19 mg/dl/min compared to 0.47 mg/dl/min in the human (Gubala and Zuba, 2003). Therefore, rats may require longer periods of exposure to achieve comparable hair levels of FAEE. In the current experiment FAEE hair levels in the rat were much lower than those typically seen in humans, but this is non-consequential as levels were still measurable to examine our primary end point.

A relationship between blood ethanol and hair FAEE was not observed in the current study as has been seen in a previous study for both the guinea pig and humans (Kulaga *et al.*, 2006). The present experiment was tightly controlled to achieve equal blood ethanol levels among laboratory animals; therefore, there may not have been enough variation in the blood ethanol concentration in order to detect such a correlation.

Also noteworthy is the fact that the profile of FAEE incorporation in hair did differ in rats as compared to humans, FAEE levels being highest for ethyl myristate, followed by

ethyl stearate, palmitate, and then oleate in the rat, whereas in humans, levels of ethyl oleate predominate followed by ethyl palmitate, stearate and myristate (Kulaga *et al*., 2006). The difference in FAEE profile among species likely reflects a natural difference in fatty acid composition. Such differences also occur between matrices such as meconium, blood and various organ tissues (Laposata and Lange, 1986; Doyle *et al*., 1994; Laposata, 1998; Klein *et al*., 1999; Bearer *et al*., 2003; Moore *et al*., 2003; Chan *et al*., 2004; Bearer *et al*., 2005) and may also occur across different ethic groups in humans. This finding stresses the importance of measuring cumulative levels of multiple FAEE as opposed to a single FAEE. Altogether, however, the LE rat appears to be a suitable model for hair FAEE research as it is sensitive enough to detect chronic alcohol use after a brief 3-week administration period.

Furthermore, an interesting finding was uncovered when we examined the relationship between post-treatment FAEE levels in 'patch' and 'no patch' hair that may contribute toward our knowledge of the mode of FAEE incorporation. For both the standardized black hair samples, and non-standardized white hair samples, cumulative levels of FAEE were found to be significantly higher post-treatment in both the 'patch' hair (hair that grew back post-treatment) and the surrounding 'no patch' hair (hair that was not shaved); however, levels in the 'patch' hair were also significantly higher compared to in the surrounding 'no patch' hair. This suggests that FAEE preferentially accumulate in actively growing hair over hair in the resting phase. Substances are incorporated into hair through blood, sweat and/or sebum, and the amount of incorporation is affected by the amount of exposure the hair receives from each of these sources, the pH of the surrounding mediums, physicochemical properties of the analyte, opportunity and ability of the analyte to penetrate the hair cells and binding sites for the analyte within the hair matrix (Kikura and Nakahara, 1998; Kintz, 2007). Previously, FAEE have been described to primarily incorporate into hair through sebum (Auwarter *et al*., 2001; Pragst and Balikova, 2006). Our results are not in opposition to this hypothesis because as the rat hair grew, it had the opportunity to interact with sebum close to the skin's surface, and this was likely a contributing source of incorporation. However, our results also suggest that blood may have been an important additional source of incorporation. Actively growing hair had higher levels of FAEE compared to the surrounding hair that had not been shaved; it did not have as much opportunity to be exposed to sebum as the static hair that was present on the rat from the beginning of the experiment. Consequently, an additional source of incorporation likely facilitated higher FAEE accumulation in actively growing hair, and this source was likely blood. Supporting this hypothesis is the fact that vascularity surrounding rat hair cells is known to dramatically increase during the hair growth phase (Durward and Rudall, 1949). Therefore, increased vascularity and blood exposure could provide a substantial increase in FAEE exposure to hair in growing follicles relative to non-growing follicles. However, this increased vascularity may also have enhanced FAEE supply to accompanying sebaceous glands, thereby also increasing FAEE exposure to growing hairs from sebum. Consequently, sebum and blood probably both act as important sources of incorporation for FAEE, a phenomenon that has not been previously documented.

In conclusion, we established the LE rat as a model to investigate the mechanism of hair accumulation of FAEE. Our results indicate that hair accumulation of FAEE is independent of melanin, thus obviating potential bias in the interpretation of chronic alcohol abuse, an issue that has marred the interpretation of certain other drugs in various ethnic groups.

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