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Inhibitory Effects of d-limonene Cleaning on the Formation of DNA Adducts in Skin and Lung of Mice Dermally Exposed to Used Gasoline Engine Oil

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피부에 폭로된 폐가솔린엔진오일로 인한 표적장기의 DNA adducts 형성과 d-라이모닌 세척효과에 대한 평가

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국문요약

작업장에서 근로자들이 엔진오일 등 각종 오일에 피부가 폭로되었을 때 이것을 쉽게 세척하기 위하여 일반적으로 솔벤트를 사용한다. 그러나 솔벤트를 사용하면 피부를 건조하게 만들 뿐 만 아니라 오일에 함유되어 있는 각종 성분들을 피부내에 흡수되는 것을 촉진시킬 수 있어서 이에대한 대처방법이 요구된다. 특히 폐가솔린엔진오일에는 방향족탄화수소(PAHs)와 같은 물질이 함유되어 있어 체내에 흡수되면 발암물질로 대사되어 표적장기(피부와 폐조직)에서 DNA adducts를 높은 수준으로 형성한다고 알려져 있다. 본 연구에서는 식물기름에서 구할 수 있는 d-라이모닌(limonene)를 세척제로 사용하여 폐가솔린엔진오일의 폭로로 인하여 형성되는 DNA adducts를 ^{32}P-postlabeling방법으로 분석함으로써 d-라이모닌의 세척효과를 평가하고자 하였다. HDC(ICR) Br 자성마우스의 견갑골 부위에 있는 털을 제거하고 그 부위에 폐가솔린엔진오일을 폭로시키고 1시간과 8시간이 지난 다음에 d-라이모닌으로 각각 세척을 하였다. 마지막 폭로를 마치고 24시간이 지난다음에 실험동물을 희생시켜 표적장기(폭로된 피부와 폐)에서 시료를 채취하였다. 먼저 시료에서 DNA를 분리하여 가수분해한 다음에 ^{32}P-postlabeling하여 DNA adducts를 분리하였다. 폐가솔린엔진오일만 폭로시킨 그룹의 피부와 폐조직에 형성된 DNA adducts가 각각 30.3±3.7과 15.7±2.4로서 대조군(2.5±1.0과 1.4±0.4)에 비하여 통계적으로 유의하게 높았고($p<0.01$), 또한 폐조직에서 보다 피부조직에서 통계적으로 유의하게 높았다($p<0.01$). 폐가솔린엔진오일을 폭로시킨 후에 d-라이모닌으로 세척한 그룹에서는 피부와 폐조직에 형성된 DNA adducts가 통계적으로 유의하게 감소하였는데($p<0.01$), 8시간 보다는 1시간이 지난 다음에 세척한 그룹에서 DNA adducts의 감소현상이 더 크게 나타났다. 결론적으로 피부에 폭로된 폐가솔린엔진오일을 d-라이모닌으로 세척하면 폐가솔린엔진오일내에 함유된 발암물질이 체내흡수되는 것이 억제되고, 피부와 폐조직 모두에서 DNA adducts의 형성을 감소시킬 수 있으며, 폐오일이 폭로된 후 빨리 세척하는 것이 더 효과적임을 증명하였다.

Keywords : d-limonene, ^{32}P-postlabeling, DNA adducts, Used gasoline engine oil(UGEO)

I. Introduction

The fresh gasoline engine oils and both the fresh and used diesel engine oils were noncarcinogenic, but used gasoline engine oils(UGEO) were carcinogenic when repeatedly applied to mouse skin(Grimmer *et al.*, 1982; Shoket *et al.*, 1989; McKee and Plutnick, 1989; Carmichael *et al.*, 1990; Carmichael *et al.*, 1992). The elevated polycyclic aromatic hydrocarbon(PAH) in used gasoline oils was a important cause. The carcinogenic su-

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bstance in UGEO result chiefly from two sources: a) fuel combustion, and b) unburned gasoline(Behn, *et al.*, 1980; Grimmer, *et al.*, 1982). About 85% of the total PAH production of an automobile engine is extracted by the lubricating oil and retained in the engine crankcase(Carmichael, *et al.*, 1990).

Because of the potential carcinogenicity of the complete mixture within UGEO, the dermal exposure UGEO usually recommended the use of hand cleanser for rapidly removing the UGEO (ATSDR, 1994). Several types of hand cleansers are currently available, some of which may contain many kinds of constituents to aid the cleaning process. But the repeated use of hand cleansers can damage the epidermal layer of the skin and result in an increased exposure(ATSDR, 1994; Carwardine, 1996). So it is very important which kind of constituents based on the cleansers. The cleaners are commonly either solvent or d-limonene based. Workers not uncommonly use the solvent meant for cleaning of tools and parts to their skin following contact. While this is not recommended because of potential of the solvent to dry the skin and act as a carrier, increasing absorption, there is needed another instead of it.

Used gasoline engine oils(UGEO) contains carcinogens and forms carcinogen-DNA adducts in skin and lung following topical application. Because the formation of adducts between DNA and electrophilic metabolites of some carcinogens in UGEO is generally accepted to be a key step in the mechanisms by which they initiate malignant transformation, this study detected the DNA adducts in skin and lung tissues of groups washed or not with d-limonene based cleanser following dermal exposure of used gasoline engine oils (UGEO).

The purpose of this study is to evaluate the effects of d-limonene for reducing the levels of DNA adducts formed in the skin and lung tissues of mice dermally exposed to Used Gasoline Engine Oil. D-limonene is a monocyclic monoterpene found in the essential oils of citrus fruits, spices and herbs, and is rapidly and extensively metabolized in mammals(Kawamori, *et al*; 1996).

For DNA adduct detection, we have applied the highly sensitive ^{32}P-postlabeling method which is based on enzymatic reactions, and is capable of detecting one adduct in 10^{7}-10^{10} normal DNA nucleotides(Guta, R.C., *et al.*, 1982; Gupta, 1985; Reddy and Randerath, 1986; Talaska, *et al.*, 1992). Washing with these cleansers were done at two different interval times following dermal application of UGEO.

II. Materials and Methods

1. Chemicals

The stock solution of used gasoline engine oil (UGEO) was provided by the American Petroleum Institute(Lot No. API 79-07). The oil had previously been used in a long term carcinogen study involving dermal applications to the shaved backs of mice, which 19 of the 51 mice dermally exposed to the UGEO developed skin tumors (Mckee & Plutnick, 1989; Tasi, 1994). The stock solution of UGEO was taken from a previously unopened container of Ashland SAE 10W40 motor oil(Ashland Petroleum Company, Ashland, KY). The d-limonene used during the animal study were provided by Gojo Industries, Inc.(3783 State road, Cuyahoga falls, OH).

2. Animal Treatment

The female HSD(ICR) Br strain mice aged 4-6 weeks were used during the completion of the animal study. The mice were purchased from Harlan Sprague Dawley(Indianapolice, IN). The animals were housed individually in plastic shoebox cages at the Kettering Laboratory animal housing facilities. The room was climate controlled at 22℃ with a 55% relative humidity. Tap water and food (Harlan Teklad LM-485 Mouse/Rat Diet 7012, Madison, WI) were provided *ad libitum*. The room's fluorescent lighting system was programmed for a 12 hour light/dark cycle beginning at 6:00 a.m. with no twilight.

The animal study design was devised following the criteria(Klaassen, *et al.*, 1995). A total of 4 animal study groups was formed; A positive control group was treated with UGEO, but the UGEO was not washed from the backs of the mice. A negative control groups was treated with kerosene which was not washed away. The

animals placed into the one study group were washed 1 hour after UGEO exposure. Those into another study group were washed 8 hours after UGEO exposure.

Dosing procedures included the application of 50 L of UGEO with a calibrated Gilson Pipetman™ to the shaved interscapular region of the mice. The mice were dosed for 5 consecutive days. Wash procedures included the application of the respective hand cleanser with a cotton swab. The cotton swab was brushed over the UGEO application area in a back and forth fashion parallel to the spinal cord for approximately 15 seconds. A cotton ball saturated with tap water was then used to remove the hand cleanser and oil residues. The mice were washed for consecutive days.

All animals were sacrificed approximately 24 hours after the last dose. Each animal was injected with a 0.4 cubic centimeter solution of pentobarbital sodium injection(NEMBUTAL® Sodium Solution, Abbott Laboratories) and water. The lungs and area of skin where dosing occurred were harvested. Each sample was placed into a labeled cryovial(Nalge Company) and immediately placed on dry ice. After harvesting procedures, all samples were stored at -80℃(Revco Freezer Model No. VLT-1786 A-O-E, Revco Scientific, Inc.).

3. DNA Isolation

DNA isolation procedures were conducted as described by Talaska *et al*(1992) with minor modifications. Approximately 0.1 g of each sample was weighed(Fisher Scientific Model XD-2200 Scale, Fisher Scientific) and placed into a 15 mL Corex test tube. A 1.0 mL solution (pH 7.4) of 1% SDS(sodium deodecyl sulfate, Sigma Chemical Co., St. Louis, MO) 1mM EDTA(ethylene diamine tetraacetic acid, Sigma Chemical Co., St. Louis, MO) was added to each tube. In addition, 24 μL of 1 M Tris(pH 7.4)(TRIZMA® Base, Sigma Chemical Co., St. Louis, MO) was added to each tube.

The contents were then homogenized with a Brinkman Model PT 10/35 homogenizer(Brinkman Instruments Co., Westbury, NJ) for 10 seconds. Then 24 μL RNase A(Ribonuclease A from bovine pancreas, Sigma Chemical Co., St. Louis, MO) and 8 μL RNase T_1(at 5 μg/μL)(Ribonuclease T_1 from *Aspergillus oryzae*, Sigma Chemical Co., St. Louis, MO) were added to each tube followed by vortexing and incubation at 37℃ for 1 hour. To each sample, 60 μL proteinase K(Tritirachium Album, STRATAGENE® Cloning Systems, La Jolla, CA) was added and the homogenate was incubated at 37℃ for 30 min. Each sample was then extracted with an equal volume of 24 mM tris-saturated phenol, then with phenol-chloroform isoamyl alcohol and finally with chloroform isoamyl alcohol(24:1). The DNA was precipitated by addition of 10 μL glycogen(at 30 μg/μL), 0.1 m*l* of 4 M lithium chloride, then 2.5 mL of 100% ice-cold ethanol. The DNA which were placed in the -80℃ freezer for 15 min, centrifuged 7,000 rpm at 4℃ for 10 min. The resultant pellet was washed briefly with 70% ethanol and air dried. The DNA pellet was dissolved in a 1% SSC/EDTA(sodium chloride, sodium citrate, ethylenediamine tetraacetic acid) solution and analyzed with a DU Series 70 Spectrophotometer(Beckman Instruments, Inc., Fullerton, CA). To calculate the DNA concentration expressed as g/μL, the absorbance at 260 nm was multiplied by 3.

4. ^{32}P-Postlabeling analysis

DNA was hydrolyzed to 3'-phosphodeoxynucleotides by incubation with micrococcal endonuclease and spleen phosphodiesterase for 3 hours. For the nuclease P_1 enhancement, the cocktail which consisted of 21.6 μL nuclease P_1 (at 5 μg/μL), 32.4 μL zinc chloride(0.3 mM) and 54.4 μL sodium acetate(0.25 M, pH 5.0), was added to each sample which were then incubated for 75 minutes. The DNA was ^{32}P-Postlabeling by incubation with 5.0 μL PNK(at 10 units/μL, T_4 polynucleotide kinase), labeling buffer(PNK and bicine) and 50 μCi/μL [γ-^{32}P]ATP for 40 minutes. The samples were spotted on PEI(polyethylene imine) cellulose plates and chromatography performed as previously described(Talaska *et al.*, 1992). The TLC plates were exposed to film into the freezer(-80℃) for approximately 72 hours. The resultant autoradiographs were used as templates to excise radioactive spots on the TLC plates as well as excising a similar sized "spot" as

a background sample.

5. Qunatitation of DNA adducts

The levels of radioactivity on spots were analyzed by Cerenkov counting in a scintillation counter(TRI-CABR® Model 2200 CA Liquid Scintillation Analyzer). DNA adducts of UGEO were determined by calculating relative adduct labeling(RAL) through the use of the formula below.

$$RAL = \frac{\text{Count per Minute of Each Adduct}}{1.25 \times 10^6 \text{ cpm/pmol ATP} \times (3240 \text{ pmol of dNP/}\mu\text{g DNA}) \times \mu\text{g DNA}} \times 10^9$$

Samples were selected for analysis using a stratified block randomized protocol to minimize bias due to run-to-run variability in the assay, and analyzed in duplicate(at least). The replicates values were within 20%.

6. Statistical analysis

Data was entered and collected in a Lotus 123 spreadsheet. The data was transferred to ASCI format and statistically analyzed using a statistical analysis software(PC SAS) program.

For testing the statistical significance, ANOVA and Tuckey test was used among study groups, and t-test was used between two groups.

III. Results

The total DNA adducts in skin and lung tissues of group which was dermally exposed to used gasoline engine oil(UGEO), were statistically significantly higher as 30.3±3.7 and 15.7±2.4, respectively, than the negative control groups in 2.5±1.0 and 1.4±0.4($p<0.01$). Fig. 1 showed the results of it, and that the total adduct level of skin was significant higher than those of lung in case of positive control group($p<0.01$).

Fig. 2 depicts the DNA adduct levels in skin tissues of treatment groups. Total DNA adducts levels in groups washed with d-limonene was all statistically significantly decreased in comparison to positive control group($p<0.01$). Of which DNA adduct was more statistically significantly decreased at 1 hour($p<0.01$) than at 8 hour washing post exposure.

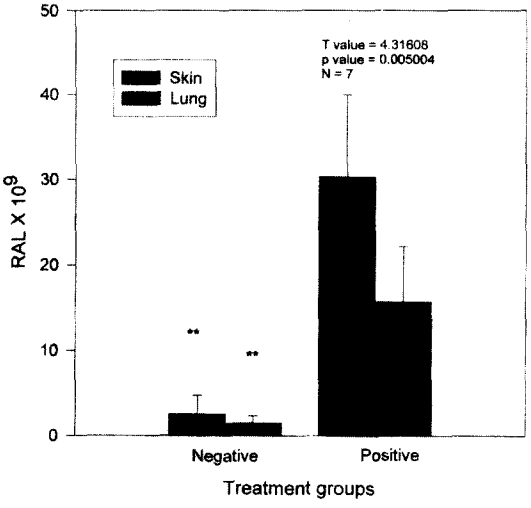

Fig. 1. Total adducts in tissues for negative and positive groups following dermal exposure of used gasoline engine oil(UGEO). Error bars show standard deviations. **Significantly different from positive group at $p<0.01$.

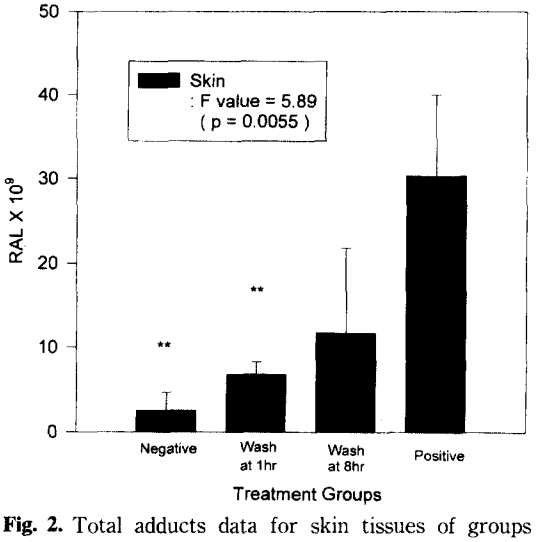

Fig. 2. Total adducts data for skin tissues of groups washed or not with Limonene following dermal exposure of used gasoline engine oil (UGEO). Error bars show standard deviations. **Significantly different from positive group at $p<0.01$.

Fig. 3 depicts the DNA adduct levels in lung tissues of treatment groups. When washed with d-limonene, the total adduct levels in lung were

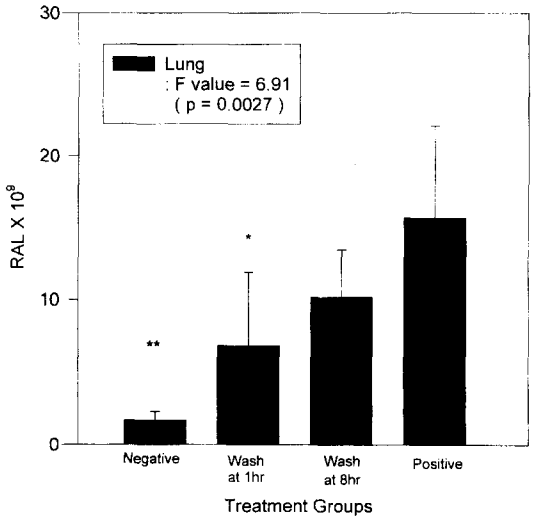

Fig. 3. Total adducts data for lung tissues of groups washed or not with Limonene following dermal exposure of used gasoline engine oil (UGEO). Error bars show standard deviations. *Significantly different from positive group at $p<0.05$, **Significantly different from positive group at $p<0.01$.

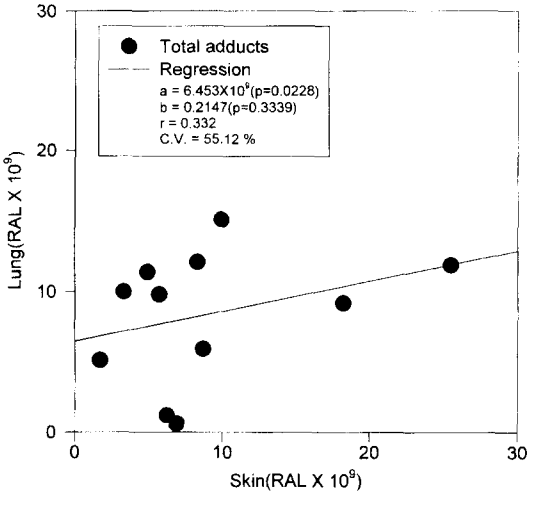

Fig. 4. Regressions of total adducts between skin and lung tissues of groups washed with Limonene following dermal exposure of used gasoline engine oil(UGEO).

statistically significantly decreased($p<0.01$), and of which at 1 hour washing was significant less than positive control group($p<0.05$). The formation of DNA adducts in lung was decreased by washing with limonene cleanser.

Fig. 4 is regression between DNA adducts in skin and lung tissues of all individual animals. The slope of regression is very gently up as 0.2147.

IV. Discussions

The used gasoline engine oils(UGEO) is carcinogenic in a long term animal studies and capable of increasing the number of carcinogen-DNA adducts in a short term animal studies when mice are dermally exposed, and the polycyclic aromatic hydrocarbon(PAH) presented in UGEO are responsible for the carcinogenicity of UGEO(Grimmer *et al.*, 1982; Shoket *et al.*, 1989; McKee and Plutnick, 1989; Camichael *et al.*, 1990; Camichael *et al.*, 1992). Carmichael, *et al* (1990) reported a study which mice were dermally exposed to UGEO, and have shown that the levels of carcinogen-DNA adducts were linearly correlated with tumorigenic responses, and the use of DNA adduct levels was proven to be a useful biomarker for assessing carcinogenic risk. Carmichael, *et al*(1992), in another study, suggested that some of the major adducts produced by dermal application of UGEO to mice are formed by reactive metabolites of benzo[g,h,i] fluoranthene, benzo[a]anphtho[1,2-d]thiophene, benzo[c]phenanthrene, benzo[g,h,i]fluoranthene, benzo[a]pyrene and benzo[g,h,i]perylene, concluded that the majority of genotoxic activity of UGEO appears to be induced by PAHs of four or fewer fused aromatic rings. Grimmer, *et al* (1982) conducted a study which involved the fraction of UGEO, and concluded that PAH were responsible for 69.4% of the total carcinogenicity of UGEO sample, and benzo[a]pyrene was responsible for 18% of the total carcinogenicity attributed to PAH.

Target organs of mice dermally exposed UGEO were skin and lung. Shoket, *et al*(1989) suggested that carcinogen-DNA adducts were significantly found in the skin and lung tissues of the mice treated with UGEO. The results of this study also showed that DNA adducts of skin and lung tissues in positive group were significantly

higher than those in negative group($p<0.01$), and then adduct level of skin tissues was significantly higher that of lung tissue($p<0.01$). This finding would indicate that the skin was able to metabolize a greater amount of the PAH as the compounds were being absorbed. The adducts level of lung tissue was lower in this group indicating that less of the PAH were passing through the skin and entering the circulatory system.

The primary recommended practices for reducing human exposure to UGEO involve the use of hand cleansers(ATSDR; 1994). The cleansers are either solvent or d-limonene based. Solvent is a popular method for workers to cleanse exposed areas of skin, but it can dry the skin and produce dermatitis following repeated skin contact which can lead to increased dermal absorption of compounds. Dobson(1979) reported the results which a cleanser free of volatile organic solvents appears to be less damaging to the barrier layer and may represent an advance in the prevention of industrial hand dermatitis. d-limonene, not solvent, is a monocyclic monoterpene found in the essential oils of citrus fruits, spices and herbs, and is rapidly and extensively metabolized in mammals(Kawamori, *et al*; 1996). The data from this study show that d-limonene statistically significantly decreased DNA adducts in all the skin and lung tissues. So the increasing rate of DNA adducts in lung tissues against that in skin tissues was very gently up(slop=0.215). It means the repeated application of d-limonene may not only remove the carcinogen of UGEO on skin, but also decrease dermal absorption into the lung, and d-limonene have done the blocking activity against forming DNA adducts by PAH or other carcinogen of UGEO in lung. Kawamori, *et al.*(1996) reported that d-limonene treatment in the drinking water was rapidly and extensively metabolized in mammals, has no toxicity, and possessed an impressive array of anti-tumorigenic activity. Some studies also reported that d-limonene has chemopreventive activity against spontaneous and chemically induced tumors in mammary gland(Elson, *et al.*, 1988; Maltzman, *et al.*, 1989), skin(Elegbede, *et al.*, 1986), liver (Dietrich and Swenberg, 1991), colon(Kawamori, *et al*; 1996), lung and forestomach of rodents (Wattenberg, *et al.*, 1989; Wattenberg and Coccia, 1991).

Ideally, a skin cleanser should be both effective and noninjurious even after repeated usage. However, this has been difficult to achieve since, in general, the greater the capability of a skin cleanser to remove and dissolve dirt or hazardous substance, the greater its potential to produce skin damage. But d-limonene was close to ideal cleanser with reference to our results. Althought our data are limited and studies on mechanism are obviously required, our findings indicate that d-limonene based cleanser appears to have a blocking activity against forming DNA adducts in skin and lung for dermally exposed with UGEO, and it is better when cleaned as possible as early after exposure. So it may represent an advance in the prevention of skin and lung cancers caused by UGEO.

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