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Effects of Purified Glycerol from Biodiesel on *Cyp1a1* and *Cyp2e1* Expressions in CBA/CA Mice

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Abstract. *Background: The glycerol from biodiesel as a valuable by-product can be used as food supplements in feed for animals. We investigated the effect of this by-product on metabolism in mice. Materials and Methods: CBA/CA mice were administered purified glycerol products (called SZME3) for 3, 6 and 24 hours. After dietary exposure, gene expressions of cytochrome P450, family 1, subfamily a polypeptide 1 (Cyp1a1) and cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1) were analysed in liver. Results: SZME3 induced an up-regulation of both genes in both genders after three hours' administration. After longer administration of SZME3, the expression of genes became similar to that of the controls. Conclusion: Based upon our data SZME3 has no long-term effect on oxidative metabolism, it seems not to cause oxidative stress for liver cells.*

Climate changes and depletion of the global oil reserves require sustainable energy production. Most developed countries are interested in new technologies to exploit renewable energy sources, and one of the most promising steps forward is that regarding biofuel production.

Biodiesel is refined from oils or fats of plant origin by transesterification and it is common biofuel in Europe (1, 2). The usable by-product of transesterification is glycerol. Several studies have evaluated the use of biodiesel glycerol in diets for poultry, pigs and ruminants, and based on their data, it represents a natural and proper alternative energy resource for animal nutrition (3-4). In animal nutrition, the carcinogenic effects and the process of biotransformation of methanol residues and trace unsaturated and polycyclic components in biodiesel glycerol is a real question for nutritional scientists. In our previous investigations in this field, we examined the biological effects of two different fractions of purified biodiesel glycerol on early inducible transcription of genes that play a central role in inflammatory and DNA damage activated signal transduction *Nfκb1* (nuclear factor kappa-light-chain enhancer of activated B-cells 1) and *Gadd45a* (growth arrest and DNA damage-inducible protein 45 alpha). Our data suggested that biodiesel glycerol fractions with higher purity have a smaller effect on apoptotic signalling and thus on evasion of apoptosis and cell survival (5).

The process of biotransformation of biodiesel glycerol is currently being highlighted comparing biodiesel glycerol and synthetic glycerol in the field of animal nutrition. Many endogenous and exogenous substrates can cause oxidative stress in biological systems. The main catalysts in oxidative changes of substrates are mono-oxygenases of the cytochrome (CYP) superfamily and the CYP enzymes are elements of a mono-oxygenase complex. The complex contains nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor, a cytochrome P450 reductase and prosthetic groups such as flavin adenine dinucleotide and flavin mononucleotide. A simplified scheme for such oxidation is shown in Figure 1 (6).

On the other hand, cytochromes are not only involved in deactivation of xenobiotics but they can also transform chemicals to toxic products by generating reactive oxygen species (ROS) (7). Overproduction of ROS results in oxidative stress and causes damaging events in cell structures, including lipid peroxidation, protein oxidation, and DNA oxidation. Oxidative stress is involved in pathogenesis in many diseases, including cancer, cardiovascular disease, diabetes mellitus, neurodegeneration, rheumatoid arthritis, and renal disease, as well as aging (8).

In the current investigation, we focus on the higher purity fraction of glycerol from biodiesel, SZME3, and analysed gene expressions of cytochrome P450, family 1, subfamily a, polypeptide 1 (*Cyp1a1*) and cytochrome P450, family 2, subfamily e, polypeptide 1 (*Cyp2e1*). These genes encode two metabolizing enzymes responsible for the oxidative transformation of trace lipid and methanol residues of SZME3.

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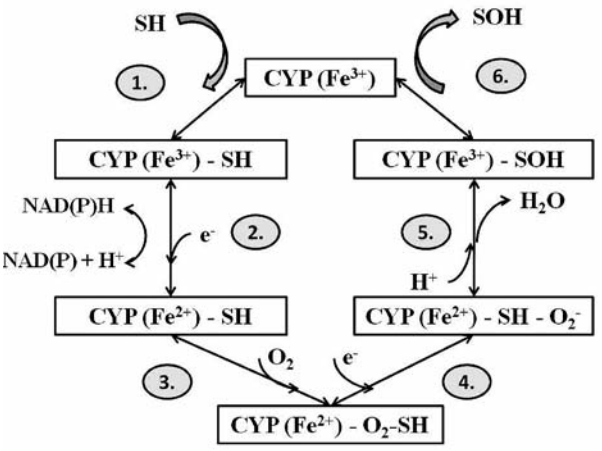

Figure 1. *Simplified scheme for CYP reactions: 1, Substrate binding. 2, Reduction. 3, O_2 binding. 4, Electron transfer. 5, Hydrogen atom transfer. 6, Product formation. SH: Hydrophobic substrate: e.g. lipid, polycyclic aromatic hydrocarbons. SOH: hydrophilic substrate, e.g. methanol.*

Materials and Methods

According to the protocol at our Institute, 5-week-old CBA/CA inbred mice were fed with 10% of purified glycerol (SZME3) in their diet. The purified glycerol was manufactured by KUKK R&D Ltd, Budapest, Hungary and contains 85% glycerol, 5% vegetable oil, 2% phosphorus, 1% sodium and 2% potassium, the methanol concentration is under 0.04%. The standard rodent chew pellet was made by Szinbád Ltd., Gödöllő, Hungary (EU registration number: HU13100039). It contains: 86% dry matter, 20% crude protein, 18.20% enzyme protein, 0.97% lysine, 0.30% methionine, 0.64% cystein, 4% crude fat, 4.30% crude fibre, 1.08%, Ca 0.85% P, 0.20% Na, 18,000 NE/kg vitamin A, 1,000 NE/kg vitamin D, 75 mg/kg vitamin E.

Mice received humane care and the experiment was carried out under the approval of the Institutional Revision Board.

Four groups of mice were maintained of both genders, containing six animals in each. Animals in group one were given SZME3 diet for 3 hours, group two for 6 hours and group three for 24 hours. The fourth group was the control; control animals consumed only the standard chew pellet. After administration, animals were sacrificed by cervical dislocation and livers of the animals were removed during autopsy. The livers were homogenized and pooled by group, then total cellular RNA was isolated from the tissues with MagNA Pure Compact automatic nucleic acid isolation system (Roche, Berlin, Germany) according to the manufacturer's instructions. The quality of the isolated RNA was checked by absorption photometry at 260/280 nm. Optical density of the RNA was between 1.9 and 2.1.

Total RNA with high purity was used in quantitative real-time PCR using a LightCycler 2.0 instrument (Roche). Reverse transcription and amplification was carried out with one-step LightCycler RNA Amplification kit (Roche) containing SYBR green fluorescent labelling. The PCR reactions were carried out in glass capillaries. The reaction mix contained 8 μl H_2O, 4 μl Light Cycler RT-PCR Reaction Mix SYBR Green, 3 μl Resolution solution, 1.6 μl $MgCl_2$, 0.4 μl Light Cyler RT-PCR Enzyme Mix, 2 μl of the primer mix and 1 μl sample RNA. The PCR parameters were: reverse transcription of 1 cycle at 55˚C for 10 min, denaturation of 1 cycle at 95˚C for 30 s, with amplification of 45 cycles of denaturation at 95˚C for 0.001 s, annealing at 55˚C for 15 s and extension at 72˚C for 4 s. Melting curves were determinied using 1 cycle: denaturation at 95˚C for 0.001 s, annealing at 55˚C, 30 s and melting at 95˚C for 0.001 s, continuous detection mode.

Primers for *Cyp1a1, Cyp2e1* and *Hprt* were selected by the primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary): *Cyp1a1* forward: 5'-CTACAGGACATT TGAGAAGGGC-3', reverse: 5'-AGGTCCAAAACAATCGTG ATGAC-3'; *Cyp2e1* forward: 5'-CGTTGCCTTGCTTGTCTGGA-3', reverse: 5'-AAGAAAGGAATTGGGAAAGGTCC-3'; *Hprt* forward: 5'-TCCTCCTCAGACCGCTTTT- 3' (19mer), reverse: 5'-CCTGGTTCATCATCGCTAATC-3' (21mer).

Fluorometric detection was carried out at 530 nm according to SYBR green fluorescent labelling. All PCR reactions were carried out in triplicates in separate runs. Absolute mRNA content of the tissues for *Cyp1a1, Cyp2e1* and *Hprt* were determined, averaged and gene expressions were calculated relative to the expression of *Hprt*.

Statistical evaluation was carried out by one-way analysis-of-covariance (ANOCVA) followed by regression analysis. STATA Release 11 software for Windows (StataCorp LP, TX, USA) was used for the analysis using the gene expression in control groups. Values of $p<0.05$ were considered to be statistically significant.

Table I. *Results of statistical analysis. Gene expression in the control group was taken as the level of regression after the one-way analysis of variance between groups.*

	95% CI		*p*-Value
Cyp1a1			
Female			
3 h	3.075	4.498	0.000
6 h	–0.345	1.078	0.269
24 h	–0.625	0.798	0.789
Male			
3 h	1.744	3.362	0.000
6 h	–0.299	1.319	0.184
24 h	–0.492	1.126	0.393
Cyp2e1			
Female			
3 h	5.270	13.050	0.001
6 h	1.870	9.650	0.009
24 h	–3.410	4.370	0.783
Male			
3 h	5.072	7.160	0.000
6 h	0.690	2.780	0.005
24 h	–1.780	0.300	0.141

CI: Confidence interval.

Results

According to our results (Table I), the expression of *Cyp1a1* and *Cyp2e1* in liver was significantly higher at the three-hour time point in both genders. *Cyp2e1* overexpression was more marked in both sexes reaching a four-fold overexpression in

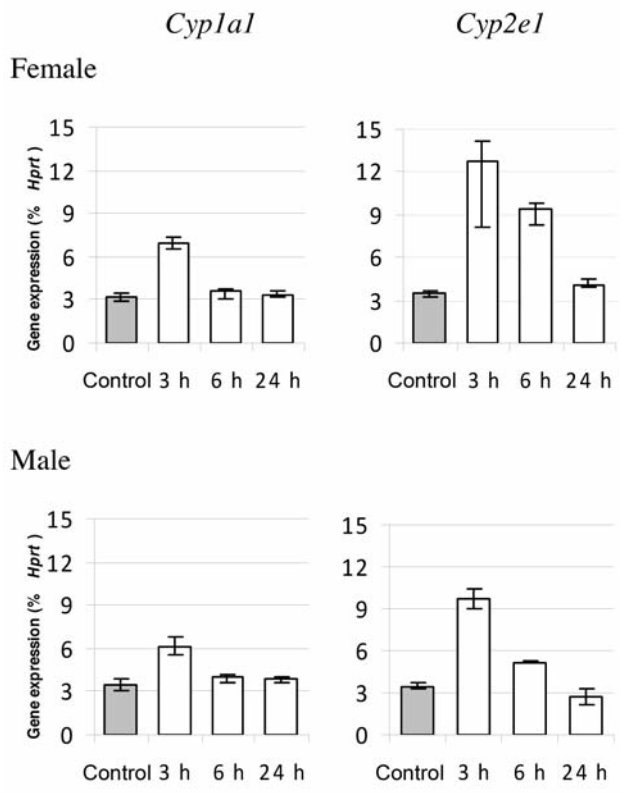

Figure 2. *Cyp1a1 and Cyp2e1 gene expression in liver of mice after 3, 6 and 24 hours SZME3 diet compared to controls. The graphs represent the mean value of expressions from the replicates.*

female mice and a three-fold in the males. At the six-hour time point *Cyp1a1* expression returned to the control's level while *Cyp2e1* tended to decrease but remained significantly up-regulated in female mice, reaching a non-significant level in the males. Figure 2 shows that the up-regulation of the genes returned to the expression levels of the controls within 24 hours from administration.

Discussion

Purified biodiesel glycerol bioutilisation and biological effect *in vivo* strongly depend on the impact of the trace element, saturated and unsaturated fatty acid and methanol content of the product. Metabolism of residual lipids and the minimal methanol contamination due to the transesterification technology is dominantly ruled by two members of the CYP superfamily: CYP1A1 and CYP2E1.

Cyp1a1 encodes a protein which localises in the endoplasmic reticulum. Endogenous substrates of *Cyp1a1* are steroids and fatty acids, and it also takes part in metabolism of caffeine, pethidine, phenacetin, progesterone and other steroids and polycyclic aromatic hydrocarbons. *Cyp2e1* localises to endoplasmic reticulum also, and is involved in metabolism of endogenous substrates such as acetone and acetol, as well as exogenous substrates for example, acetaminophen, halothane, isoflurane, paracetamol, benzene, aniline, nitrosamines, ethanol and methanol (9). In addition, in rodents, alcohol dehydrogenisation is also maintained mostly by Cyp2e1 and less by the alcohol dehydrogenases (10). Levels of *Cyp2e1* are elevated under a variety of physiological and pathophysiological conditions, such as alcohol and xenobiotic exposure. Cyp2e1 is an effective generator of ROS, such as the superoxide anion radical and hydrogen peroxide, and in the presence of iron catalysts produces powerful oxidants such as the hydroxyl radical (9). Lu *et al*. demonstrated on *in vivo* rodent liver models that Cyp2e1-derived oxidative stress may inhibit oxidation of fatty acids, resulting in steatotic hepatic lesions (11).

According to our results the expressions of both *Cyp1a1* and the *Cyp2e1* were elevated after supplementing the animals' diet with SZME3, as a response to SZME3 exposure due to its variety of lipid contaminants and methanol. This up-regulation quickly subsided and expressions equilibrated with those of the control within six hours, suggesting that the pathway of biotransformation adapted to the metabolic needs. No significant alterations were seen after six hours, nor at the 24-hour time points for either of the genes. Sex differences in expression could occur due to the fact that the microsomal density of Cyp2e1 in female mice is higher as activity of microsomal mono-oxygenase activity is modified by sex hormones (12).

Based upon our data, SZME3, the natural purified glycerol fraction evaluated here seems to have only a transient short-term effect on these two genes, which is lost within 24 hours, putting a minimal and reversible exposure burden on the metabolic balance of the animals.

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