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Feeding Purified Glycerol from Biodiesel to CBA/CA Mice: Effects on *Gadd45a* and *Nfκb1* Expressions

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Abstract. *Background: The turn towards renewable energy sources has increased the production of biodiesel from rapeseed oil, leaving glycerol as a valuable by-product. Several studies have evaluated this product in feed for poultry, swine and ruminants. We investigated the effect of these glycerol products on the expression of DNA damage-inducible genes in mice. Materials and Methods: CBA/CA mice were administered two different purified glycerol products (SZME2, SZME3) for 3, 6 and 24 hours. After dietary exposure, gene expressions of nuclear factor kappa-light-chain-enhancer of activated B-cells 1 (NFkb1) and growth arrest and DNA-damage-inducible protein 45 alpha (Gadd45a) were analysed. Results: SZME2 induced a down-regulation of the two genes in all investigated organs, including the bone marrow in both genders. After administration of SZME3, up-regulation of the two genes was observed in bone marrow in males, and the up-regulation of Gadd45a in liver, also in males. Conclusion: Based upon our data, SZME3, which contains glycerol of higher purification, did not induce down-regulation in genes which are involved in apoptosis.*

In the European Union, the turn towards renewable energy sources has increased the production of biodiesel from rapeseed oil. Biodiesel fuel is an alternative energy source, which may help to reduce air pollution, as well as our dependence on petroleum for energy.

Several processes have already been developed for the production of biodiesel (1-4). The rapid growth of ethanol and biodiesel production has raised questions about possible uses for the by-products. One by-product of biodiesel production is glycerol, the carbohydrate fraction that makes up about 10-11% by weight of typical triglycerides. Glycerol is a natural liquid substance registered in the European Union as a feed additive, E422. Several studies have evaluated the use of glycerol in diets for poultry, pigs and ruminants (5-10). Researchers at the University of Arkansas Centre of Excellence for Poultry Science examined the possibility of using glycerol as a dietary supplement in growing broiler chickens. In a short-term preliminary trial, Waldroup *et al*. found that as much as 10% glycerol could be fed to chickens up to 16 days of age without any detrimental effects on growth performance or meat quality. While 5% glycerol had no effect on body weight, feed intake, feed conversion or mortality, 10% glycerol affected feed flow rate (in the feeders used), reducing feed intake and consequently reduced body weight. Although the results suggest that glycerol can be used as an energy source in broiler diets, Waldroup *et al*. cautioned that additional research was needed to evaluate the quality of the glycerol used and its effect on feed texture and pellet quality (7, 11).

Synthetic glycerol represents basically the energy source in the diet whereas purified glycerol fractions from biodiesel contain trace amounts of a variety of different other chemical components: generally tocoferol antioxidants, potassium and methanol in minute amounts. Methanol has already been proven to induce DNA damage *via* its dehydrogenization into formaldehyde, whereas tocoferol dietary antioxidants showed several beneficial effects on the multistep process of carcinogenesis and on the extrinsic apoptotic pathways (12).

In our study, we aimed to analyse the effects of SZME2 and SZME3 on the gene expression of the early responding transcriptional activator nuclear factor kappa-light-chain-enhancer of activated B-cells 1 (*Nfkb1*), which plays crucial role in the regulation of apoptosis and cell death. We also investigated the expression of growth arrest and DNA-damage-inducible protein 45 alpha (*Gadd45a*) that is a single-strand DNA damage-inducible gene in tight signalling connection with the NFκB pathway. SZME 2 and SZME3 are two promising, neutralized, filtered and distilled glycerol fractions of biodiesel, with acceptably high purity and with methanol content lower than 0.1%.

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Table I. *Chemical composition of standard rodent chow pellet. Ingredients: corn, wheat, sunflower, soybean meal, milk powder, yeast, lime, meal, sunflower oil (Manufactured by: Szinbád Ltd., Gödöllő, Hungary; EU registration number: HU13100039).*

Energy	11MJ/kg
Dry matter	86%
Crude protein	20%
Enzyme protein	18.20%
Lysine	0.97%
Methionine	0.30%
Cystenine	0.64%
Crude fat	4%
Crude fibre	4.30%
Ca	1.08%
P	0.85%
Na	0.20%
Vitamin A	18000 NE/kg
Vitamin D	1000 NE/kg
Vitamin E	75 mg/kg

Table II. *Chemical composition of SZME2 and SZME3 (Manufactured by: KUKK R&D Ltd, Budapest, Hungary).*

	SZME2	SZME3
Glycerol*	60%	85%
Vegetable oil	20%	5%
Phosphorus**	4%	2%
Sodium**	1%	1%
Potassium**	5%	2%
Methanol***	0.04%	0.04%
Water and other mineral components	9.96%	9.96%

*High performance liquid chromatography; **inductively coupled plasma spectrometry; ***gas chromatography.

Materials and Methods

Five-week-old CBA/CA inbred mice (weighing between 20-25 g) were fed with standard rodent chow pellet, SZME2 or SZME3. SZME2- and SZME3-containing diet were prepared by adding SZME2 or SZME3 at the rate of 10% dry matter content of the chow pellet. Nutritional and chemical composition of the administered standard rodent chow pellet and the purified glycerol fractions SZME2 and SZME3 are presented in Table I and Table II.

Animals were treated humanely and the experiment was carried out under the approval of the Institutional Revision Board.

Seven groups were maintained in both genders, containing six animals in each. Animals in group one were given SZME2 diet for 3, in group two for 6 and in group three for 24 hours. SZME3 diet was given to the fourth group for 3, to the fifth for 6 and to the sixth for 24 hours. The seventh group was the control; control animals consumed only the standard chow pellet. After administration animals were sacrificed by cervical dislocation and liver, spleen and bone marrow of the animals were removed during autopsy. The organs were homogenized and pooled by group, then total cellular RNA was isolated from the organs 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 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 in 20 μl final volume. The reaction mix contained: 8 μl water, Light Cycler RT-PCR Reaction Mix SYBR Green: 4 μl, Resolution solution: 3 μl, $MgCl_2$: 1.6 μl, Light Cyler RT-PCR Enzyme Mix: 0.4 μl, 2 μl of the primer mix and 1 μl sample RNA.

Primers for *Nfκb1, Gadd45a* 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) and were the following: *Nfκb1* forward: 5'CACTGCTCAGGTCCACTGTC 3' (20mer), reverse: 5'CTGT CACTATCCCGGAGTTCA3' (21mer); *Gadd45a* forward: 5'CTGCCT CCTGGTCACGAA 3' (18mer), reverse: 5'TTGCCTCTGCTCTC TTCACA 3' (20mer); *Hprt* forward: 5'TCCTCCTC AGACCGCTTTT 3' (19mer), reverse: 5'CCTGGTTCATCATCG CTAATC3' (21mer).

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: 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.

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 *Gadd45a, Nfκb1* and *Hprt* were determined, averaged and gene expression alterations were calculated relative to expression of *Hprt*.

Results

Expressions of the investigated genes showed obviously higher gene expressions generally in SZME3 administration than in SZME2, but showed lesser differences compared to the expressions of the control groups (Figure 1).

SZME2 administration reduced the *Nfκb1* expression to nearly half that of the control expression, and this down-regulation of *Nfκb1* expression was seen in both males and females for all exposure times. SZME2 consumption in male mice resulted in greater underexpression than in the females in all investigated organs and the expression suppression ranged between 47-75% of the control values at all time points. SZME3 evoked no remarkable change in any organ in female mice. *Nfκb1* expression values were similar to those of the controls' in the liver and spleen with SZME 3 treatment after 3 and 6 hours of consumption. However, in the SZME3 male groups, *Nfκb1* expressions of the spleen and the bone marrow were higher at the 24-hour time point, surpassing the level of that of the controls 57% in the spleen and 82% in the bone marrow.

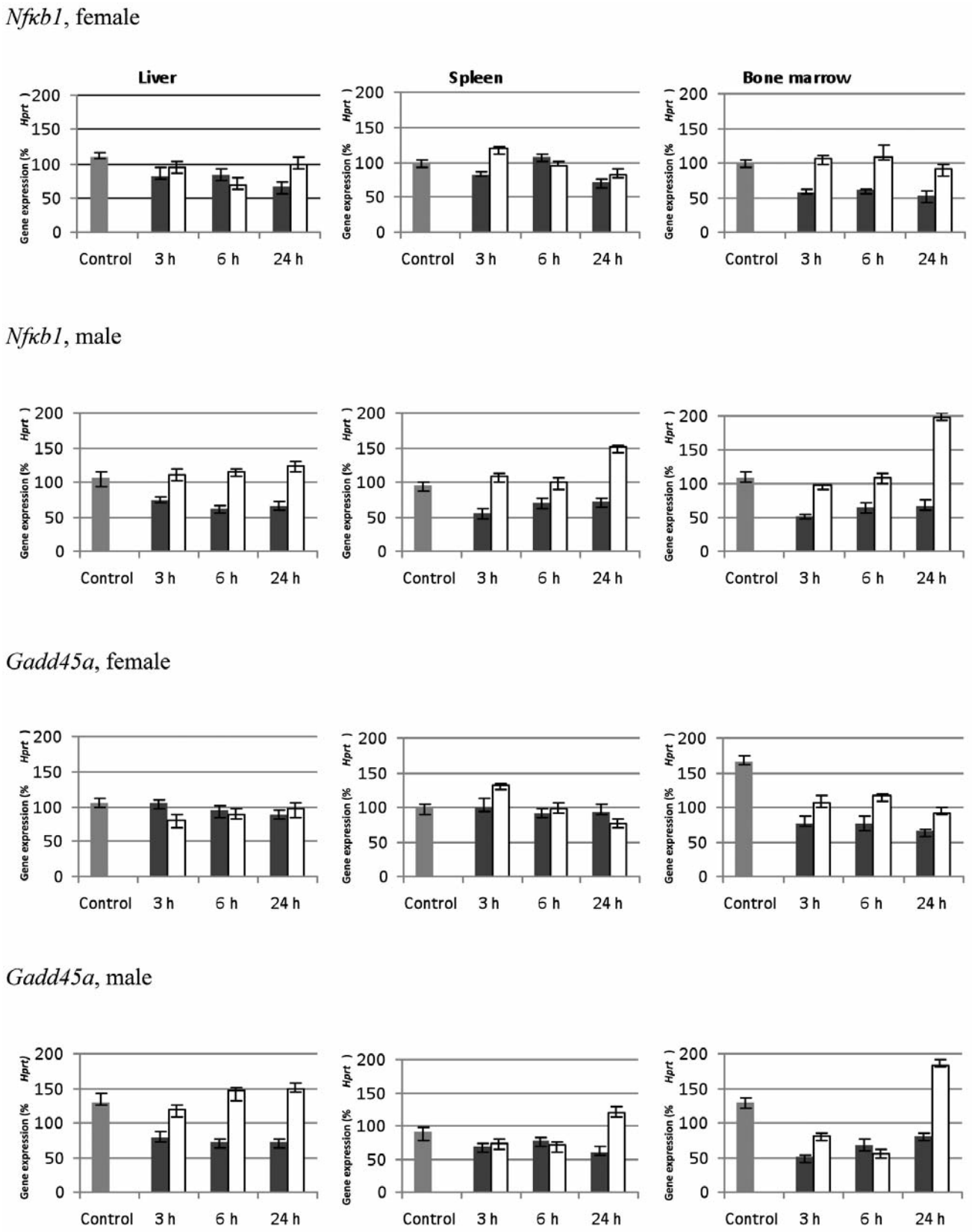

Figure 1. *Nfκb1 and Gadd45a gene expression in mice after 3, 6 or 24 hours SZME2 or SZME3 diet compared to controls (■ SZME2, □ SZME3).*

Gadd45a expression showed negligible changes, both in the liver and the spleen of female mice after SZME2 and SZME3 administration. In the liver of male mice, *Gadd45a* expression changed similarly to *Nfκb1* expression: SZME2 reduced expression around 50% at all three time points, whereas SZME3 evoked no considerable change. In the bone marrow of female mice, both SZME2 and SZME3 treatment similarly reduced the *Gadd45a* expression The reduction after SZME2 consumption was more characteristic, SZME2: 54-60%, SZME3: 30-45%. SZME2 suppressed *Gadd45a* expressions in the spleen and in the bone marrow of male mice after 3 and 6 hours of administration. This expression suppression remained between 15-33%, in the bone marrow it reached the 37-60% range. After 24 hour's nutritional exposure to SZME3, *Gadd45a* expression was seen to be elevated in the bone marrow of male mice compared to those of the controls and the SZME2 group.

Discussion

NFκB is a protein complex that acts as a transcription factor and regulates genes that control cell proliferation and cell survival and mediate the inflammatory response. The NFκB family includes homo- and heterodimers that are formed from five structure-related protein subunits: p50 (NFκB1), p52 (NFκB2), p65 (RelA), c-Rel, Rel B. NFκB can found in various types of cells. NFκB normally binds to IκB inhibitors. Stress stimulates IκB kinase, and activated IκB kinase mediates IκB phosphorylation. The NFκB-IκB complex degrades and the free NFκB translocates into the nucleus and regulates the transcription of target genes (13, 14). NFκB complex can activate different pathway, the canonical (alternative) and the non canonical (alternate) pathway, and so has apoptotic or antiapoptotic effects depending on the manner of its induction (15). The p65/RelA subunit of NFκB (NFκB1) can induce proapoptotic response in certain circumstances, and also induces other transcriptional factors, which activates cell apoptosis. Down-regulation of *Nfκb1* results in a weaker signal on cyclin D1 driven cell-cycle regulation and down-regulation of MKK4-JNK apoptosis inducing pathway *via* lower activation on the Fas/FasL transcription factor (16). These molecular events potentially facilitate survival of cells genetically altered by external chemical exposure.

Gadd45a is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. Gadd45 family members interact with the upstream kinase mitogen-activated protein kinase 4 (Mekk4) which activates JNK. The down-regulation of *Gadd45a* lead to NfκB-dependent escape from programmed cell death through the JNK cascade (17).

Administration of SZME2 caused down-regulation of *Nfkb1* and *Gadd45a* in almost every tissue, at every time point, in both genders. This down-regulation reached the remarkable value of 50% in the bone marrow of both sexes. Based upon this finding, we can conclude that implication of SZME2 in the diet results in early down-regulation of gene of the extrinsic apoptotic cascade *via* suppression of the DNA damage inducible *Gadd45a* expression and suppression of *Nfκb1*, potentiating cell survival mechanisms of the cells with damaged genetic material. SZME3 had much less effect on gene expressions of the vast majority of the investigated organs, although isolated induction of *Gadd45a* and *Nfκb1* was seen in the spleen and bone marrow of male mice was the 24-hour time point; an up-regulation peak was also noticed in the spleen of female mice at the 3-hour time point, but it was still 19% and 31%. These data suggest that glycerol fractions with higher purity have less effect on the apoptotic signalling and thus on the evasion of apoptosis and cell survival.

Our investigation underlies the necessity of animal carcinogenicity bioassays on large laboratory animal populations before introducing purified glycerol by-products into the market. SZME2 and SZME3 are purified glycerol products from the laboratory of KUKK R&D Ltd, where the aim is to develop the technology of biodiesel glycerol purification to gain a glycerol fraction that could be physiologically optimal for animal feeding, with cost-effective highest purity and with the least possible risk of carcinogenicity.

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