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Effects of Purified Glycerol from Biodiesel on miRNAs Compared to the Expression Profile of Selected mRNAs in Balb/c Mice

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Abstract. *Background: We investigated the effect of corn-derived biodiesel glycerol on microRNAs (miRNAs) and mRNAs, which play a central role in regulating cell survival, apoptosis and carcinogenesis. Materials and Methods: Inbred Balb/c mice were treated with purified glycerol from biodiesel for 24 hours. After administration, we determined the expressions of miR-21, miR-27a, miR-34a, miR-93, miR-143, miR-146a, miR-148a, miR-155, miR-196a, miR-203, miR-205, miR-221 and nuclear factor kappa-light-chain enhancer of activated B-cells-1 (Nfκb1), mitogen-activated protein kinase-8 (Mapk8) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-ras) genes in the liver of mice. Results: We found a parallel altered expression of miRNAs and mRNAs in animals consuming biodiesel glycerol that compared to control mice; these alterations reached significant levels only in few cases. Conclusion: Biodiesel glycerol presents no higher risk for carcinogenicity or toxicity.*

There are currently several companies which produce biodiesel from rapseed- or corn oil by trans-esterification. The by-product of trans-esterification is a fraction with a high percentage of glycerol. We have been examining whether the purified glycerol fraction from biodiesel is suitable for animal feeding, at the Institute of Public Health at the University of Pécs, since 2007 (1, 2).

In our previous studies, we examined the biological effects of biodiesel glycerol on two genes: nuclear factor kappa-light-chain enhancer of activated B-cells 1 (*Nfκb1*) and growth arrest and DNA damage-inducible protein 45 alpha (*Gadd45a*), which play a central role in cell survival and apoptosis. We also investigated the expression of genes for cytochrome *P450*, family 1, subfamily a polypeptide 1 (*Cyp1a1*) and cytochrome *P450*, family 2, subfamily e, polypeptide 1 (*Cyp2e1*), which encode metabolizing enzymes responsible for the oxidative transformation of trace lipid and methanol residues of biodiesel glycerol. In these 'short-term' animal studies we found that high purity biodiesel glycerol has no significant effect on the expression of *Nfκb1* and *Gadd45a*, and has only a transient short-term effect on the gene expression levels of *Cyp1a1* and *Cyp2e1* (3, 4).

Szendi *et al*. also found no toxic effect of biodiesel glycerol in different 'short-term' and sub-chronic oral toxicity tests in Long-Evans rats (5, 6).

Recently miRNAs have become the focus of molecular genomic research. miRNAs are highly conserved, small, endogenous non-protein-coding RNAs; their genes are frequently located in cancer-associated genomic regions. They are partially complementary to one or more RNA transcripts and after binding they can inhibit protein translation. Several studies have shown, that miRNAs regulate cell proliferation and apoptosis, function as oncogenes or tumour suppressors, and also have a role in immune response. miRNA expression profiles can have predictive value for assessing chemical carcinogenesis (7-9).

In this investigation, we evaluated the expression of several miRNAs (*miR-21, miR-27a, miR-34a, miR-93, miR-143, miR-146a, miR-148a, miR-155, miR-196a, miR-203, miR-205, miR-221*) in liver of mice and compared it to the expression of the following mRNAs: *Nfκb1*, mitogen-activated protein kinase-8 (*Mapk8*) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-ras*), in Balb/c mice after administration of corn-derived biodiesel glycerol.

In liver tissue *miR-21, miR-27a, miR-34a, miR-93, miR-148a, miR-155* and *miR-221* seem to be oncogenes, while *miR-146a* and *miR-203* are tumour suppressor genes (7-15). *K-ras* is known as an oncogene, NFKB is an antiapoptotic transcription factor, which regulates genes *via* the mitogen

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activated protein kinase (MKK)/c-Jun N-terminal kinase (JNK) pathway that control cell proliferation and cell survival and mediate inflammatory response (16).

Materials and Methods

Five-week-old Balb/c inbred mice were fed with purified biodiesel glycerol (SZME3) in their diet for 24 hours. SZME3 was manufactured by KUKK R&D Ltd (Budapest, Hungary) and contains 85% glycerol, 5% vegetable oil, 2% phosphorus, 1% sodium and 2% potassium and less than 0.04% methanol.

The animal diet groups were maintained with twenty-two mice, twelve males and twelve females in each. Group one was given the SZME3 diet, namely 10% SZME3 by dry weight mixed in the standard chew pellet, while group two, representing the control group, consumed standard laboratory chew pellet. The standard rodent chew pellet was manufactured by Szinbád Ltd. (Gödöllő, Hungary; 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, 18000 NE/kg vitamin A, 1000 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. During exposition, the behaviour of investigated mice was normal, they ate the same amount from SZME3-rich chew pellet as control animals from standard chew pellet, and their final weights were same. After 24 hours' administration, animals were autopsied by cervical dislocation and the livers of the mice were removed.

The livers were homogenized and pooled by group, and then total cellular RNA was isolated from the tissues with the High Pure miRNA Isolation Kit (Roche, Berlin, Germany), according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA with the cDNA Synthesis System Kit (Roche) and AMV reverse transcriptase. The concentration of miRNA was determined by absorption photometry.

cDNA from total RNA was used for miRNA expression analysis in a LightCycler 480 Polymerase Chain Reaction (PCR) System using the LightCycler 480 SYBR Green I Master Kit. The PCR reactions were carried out in 96-well plates (Real-Time Ready Custom Panel 96; Roche). The PCR reaction mix contained 3 μl H_2O, 10 μl LightCycler 480 SYBR Green I Master Mix, 2 μl of the equal mixture of the miRNA-specific forward and reverse primers and 5 μl of the sample cDNA. The PCR parameters were: pre-incubation of one cycle at 95˚C for 10 min, amplification of 65 cycles at 95˚C for 10 s, 42˚C for 20 s and 72˚C for 15 s. Melting curves were determined using one cycle: at 95˚C for 5 s, 65˚C for 30 s and melting at 97˚C in a continuous detection mode. Detection was carried out in a LC480 PCR System with the Light Cycler 480 Software (Roche). Absolute quantification was calculated for all samples with the LightCycler 480 Software using the second derivate maximum. miRNA expressions were determined using 5 S RNA as internal control.

cDNA from total RNA was also used for mRNA expression analysis of *K-ras, Nfκb1, Mapk8* and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) in a LightCycler 2.0 carousel-based PCR system, using FAM labelled Universal Probe Library probes (*Upl*; Roche) and LightCycler 480 SYBR Green I (Roche). The PCR reactions were carried out in glass capillaries in 20 μl final volume. The reaction mix contained: 5 μl sample cDNA, 1 μl of the primer, 1 μl of the UPL probe, 4 μl of LightCycler 480 SYBR Green I Master Mix and 9 μl H_2O. PCR parameters were: pre-incubation of one cycle at 95˚C for 10 min, amplification of 45 cycles of denaturation at 95˚C for 10 s, annealing at 55˚C for 40 s, extension at 72˚C for 1 s.

Primers of miRNAs were synthesized by TIB Molbiol, ADR Logistics (Roche), and are shown in Table I. Probes and primers of

Table I. *Primers and probes of investigated miRNAs and mRNAs.*

miRNA/ mRNA	Primers	Probe
miR-21		
forward:	5'-GCTTATCAGACTGATGTTGACTG-3'	
reverse:	5'-CAGCCCATCGACTGGTG-3'	
miR-27a		
forward:	5'-GCAGGGCTTAGCTGCTTG-3'	
reverse:	5'-GGCGGAACTTAGCCACTGT-3'	
miR-34a		
forward:	5'-TGGCAGTGTCTTAGCTGGTTG-3'	
reverse:	5'-GGCAGTATACTTGCTGATTGCTT-3'	
miR-93		
forward:	5'-AAGTGCTGTTCGTGCAGGT-3'	
reverse:	5'-CTCGGGAAGTGCTAGCTCA-3'	
miR-143		
forward:	5'-TGAGGTGCAGTGCTGCATC-3'	
reverse:	5'-GCTACAGTGCTTCATCTCAGACTC-3'	
miR-146a		
forward:	5'-TTGAGAACTGAATTCCATGG-3'	
reverse:	5'-GCTGAAGAACTGAATTTCAGAG-3'	
miR-148a		
forward:	5'-GAGGAAGACAGCACGTTTGGT-3'	
reverse:	5'-AAAGGCGCAGCGACGT-3'	
miR-155		
forward:	5'-TTAATGCTAATCGTGATAGGG-3'	
reverse:	5'-GCTAATATGTAGGAGTCAGTTGGA-3'	
miR-196a		
forward:	5'-TAGGTAGTTTCATGTTGTTGGG-3'	
reverse:	5'-ATCGGGTGGTTTAATGTTG-3'	
miR-203		
forward:	5'-TCCAGTGGTTCTTAACAGTTCA-3'	
reverse:	5'-GGTCTAGTGGTCCTAAACATTTC-3'	
miR-205		
forward:	5'-CCTTCATTCCACCGGAGT-3'	
reverse:	5'-GAACTTCACTCCACTGAAATCTG-3'	
miR-221		
forward:	5'-CCTGGCATACAATGTAGATTTCTG-3'	
reverse:	5'-AAACCCAGCAGACAATGTAGCT-3'	
Nfκb1		
forward:	5'-CACTGCTCAGGTCCACTGTC-3'	Upl 10
reverse:	5'-CTGTCACTATCCCGGAGT-3'	
Mapk8		
forward:	5'-AACTGTTCCCCGATGTGCT-3'	Upl 33
reverse:	5'-TCTCTTGCCTGACTGGCTTT-3'	
K-ras		
forward:	5'-TGTGGATGAGTATGACCCTACG-3'	Upl 62
reverse:	5'-CCCTCATTGCACTGTACTCCT-3'	
Hprt		
forward:	5'-TCCTCCTCAGACCGCTTTT-3'	Upl 95
reverse:	5'-CCTGGTTCATCATCGCTAATC-3'	

Table II. *Gene expression of miRNAs, using 5S RNA as internal control, in the liver of mice after the addition of SZME3 to their diet.*

miRNA	Gender	Relative expression				*p*-Value
		Control		SZME3		
		Mean	95% CI	Mean	95% CI	
miR-21	Female	354	307.5-400.5	356	284.4-427.7	0.7654
	Male	298	278.6-317.4	320	264.7-375.3	0.2382
miR-27a	Female	21.6	18.7-24.5	15.6	12.1-19.1	0.0025
	Male	13.6	10.2-17.	15.3	14.6-16.	0.138
miR-34a	Female	18.43	15.1-21.7	24.6	19.-30.2	0.0422
	Male	28.3	21-35.6	30.9	26.7-35.1	0.145
miR-93	Female	62.1	50.3-73.9	67.9	65.1-70.7	0.1110
	Male	49.8	40.1-59.5	52.9	44.9-60.9	0.0176
miR-143	Female	10.2	4.9-15.5	10.4	6.1- 14.7	0.6449
	Male	11.4	9-13.8	12.1	6.8-17.4	0.6319
miR-146a	Female	0.51	0.23-0.79	0.383	0.48-1.25	0.5154
	Male	0.58	0.38-0.78	0.59	0.33-0.85	0.86
miR-148a	Female	0.0084	0.0051-0.0117	0.0082	0.0044-0.12	0.3206
	Male	0.0157	0.0034-0.348	0.004	0.0067-0.0147	0.2149
miR-155	Female	24.9	20.6-290.2	25.3	21.6-29.1	0.1201
	Male	28.9	26.5-31.3	12.9	9.8-16	0.0002
miR-196a	Female	28.3	21.5-35.1	25.6	22.1-29.1	0.1452
	Male	49.2	42.8-55.6	46.1	41.9-50.3	0.0270
miR-203	Female	0.614	0.207-1.021	0.67	0.444-0.896	0.3178
	Male	0.616	0.335-0.897	0.798	0.673-0.923	0.0412
miR-205	Female	0.926	0.401-1.451	0.986	0.652-1.319	0.3176
	Male	0.804	0.378-1.23	0.783	0.556-1.01	0.6957
miR-221	Female	5.5	4.3-6.6	6.83	5.9-7.8	0.0109
	Male	4.5	2.9-5.9	5.43	3.9-6.9	0.0079

CI: Confidence interval.

mRNAs were designed and selected by the Roche database (www.applied-science.roche.com) and are also shown in Table I.

All PCR reactions were run in triplicates, in separate runs. The concentrations of miRNAs and mRNAs for *Nfκb1, Mapk8, K-ras* and *Hprt* were determined in liver tissue and averaged. The gene expressions of *Nfκb1, Mapk8, K-ras* were calculated relative to the expression of *Hprt*. Statistical evaluation was carried out by paired *t*-test using the STATA Release 11 software for Windows (StataCorp LP, Texas, USA). Values of $p<0.05$ were considered to be statistically significant.

Results

Male mice exhibited more significant expression changes in the investigated miRNAs than did females.

In the group of female mice administered SZME3, the oncogene *miR-27a* was significantly de-regulated in parallel to the down-regulation of oncogene *K-ras* and antiapoptotic *Nfkb1* and *Mapk8*, while two other oncogene miRNAs, *miR-34a* and *miR-221*, were found to be overexpressed.

In male mice, 24-hours' SZME3 administration resulted in the up-regulation of oncogene *miR-93a* and *miR-221*, while the oncogenes *miR-155* with *miR-196a* were also observed to be down-regulated by the under-expression of *K-ras*. In male mice *Nfkb1* expression was not altered, while *Mapk8* was also down-regulated.

The investigated mRNA exhibited significant expression changes in all investigated animals and almost in all investigated genes, while the expression of oncogenes *miR-21, miR-143, miR-148a* and *miR-205* and tumour suppressor *miR-146a* in the investigated animals, was similar to control. The results are shown in Tables II and III.

Discussion

Many studies have examined the expression profiles of miRNAs in different tumour types. The role in tumourigenesis, of the miRNAs examined in the present investigation, can be seen in Table IV.

Karakatsanis *et al*. examined patients with hepatocellular carcinoma, and found elevated expression of *miR-21, miR-221,* and up-regulation of *miR-146a* (10). Ji *et al*. investigated the oncogenic effect of *miR-27a* in rat liver (11).

Table III. *Gene expression of mRNAs relative to expression of hypoxanthine guanine phosphoribosyl transferase (Hprt) (%) in the liver of mice after addition of SZME3 to their diet.*

mRNA	Gender	Relative expression				*p*-Value
		Control		SZME3		
		Mean	95% CI	Mean	95% CI	
Nfκb1	Female	170	152.6-187.4	123.7	94.9-152.5	0.0041
	Male	126.7	105.8-147.5	120	111-128	0.153
Mapk8	Female	86.7	73.9-99.4	65	65-65	0.0182
	Male	87	73.9-100.2	59.3	46.8-71.8	0.0006
K-ras	Female	116	116-116	88.7	71.8-105.6	0.0201
	Male	94	81.6-106.4	84	72.6-95.4	0.033

CI: Confidence interval.

Table IV. *miRNA regulation in cancer.*

Cancer	Up-regulated (Ref)	Down-regulated (Ref)
Leukaemia, lymphoma	*miR-155* (8)	
Breast cancer		*miR-155* (7)
Colorectal cancer	*miR-21* (7), *miR-221* (20)	*miR-34a* (7)
Brain cancer	*miR-21*, *miR-221* (8)	
Head and neck cancer	*mir-21*, *miR-155* (9)	
Hepatocellular carcinoma	*miR-21* (7-10), *miR-34a*, (9)	*miR-146a* (10),
	miR-93 (13), *miR-221* (10),	*miR-203* (14)
	miR-27a (11), *miR-148a* (15), *miR-155* (12)	
Neuroblastoma		*miR-34a* (17)
Pancreatic cancer	*miR-21, miR-221* (7)	
Thyroid cancer	*miR-146a, miR-221* (7, 8)	

Welch *et al*. found that *miR-34a* functions as a potential tumour suppressor in neuroblastoma cells, as it directly targets the mRNA encoding the E2F3 protein, a potent transcriptional inducer of cell-cycle progression, while *miR-34a* is an oncogene in liver tissue (9, 17). Wang *et al*. found that the expression of *miR-155* was significantly higher in the liver tissue of mice after a choline-rich diet. Choline presence indicates an inflammatory response and exposure to the agent *via* animal diet causes hepatocellular carcinoma (12). Tsai *et al*. analyzed gastric cancer samples, and their data suggest that *miR-196a* is an oncogene, since the expression level of *miR-196a* was significantly increased in primary gastric cancer tissues (18). Fang *et al*. found that miR-93 enhances cell survival, while promoting tumour growth (13). *miR-203* was found to be a tumour suppressor miRNA in hepatocellular carcinoma, while it is up-regulated in several other types of tumours, for example breast, colorectal and ovarian cancer (14). *miR-148a* is also a tumour suppressor, it suppresses cell proliferation, cell progression and migration (15).

NFKB is a protein complex and has a central role in the regulation of cell proliferation, differentiation and apoptosis, as well as of many miRNAs. Ma *et al*. found a positive correlation between *miR-34a, miR-155* and *miR-221* up-regulation and *Nfκb* activation (19). The ret proto-oncogene (RET)/patched (PTC)/rat sarcoma virus oncogene (RAS)/v-raf murine sarcoma viral oncogene homolog B1 (BRAF) signalling pathway has a central role in papillary thyroid carcinoma, while *miR-221*, *miR-222* and *miR-146* were also strongly overexpressed in thyroid tumours compared with unaffected thyroid tissues (20). The MAPKs are a family of serine-threonine protein kinases that participate in major signalling systems, as they are also involved in the NFKB and RAS pathways. According to Paroo *et al*., the miRNA-generating complex is regulated by MAPK/ERK and this regulation is important in effecting mitogenic signalling; they demonstrated a direct connection between a cell signalling pathway and the core miRNAs (21).

In our investigation, the underexpression of antiapoptotic *Nfκb1* and *Mapk8*, and oncogene *K-ras* coexisted with a lower

concentration of oncogenes *miR-27a, miR-155* and *miR-196a*, and with a higher concentration of tumour suppressor *miR-203*. There was no significant alteration in the expression of oncogenes *miR-21, miR-143, miR-148a* and *miR-205* and tumor suppressor miR-146a. These data suggest that biodiesel glycerol may have a positive effect on the down-regulation of oncogene *K-ras* and by maintaining a low expression of *Nfκb1* and *Mapk8* it can facilitate the death of demaged cells. However the expression of oncogenes *mir-34a, miR-93* in female mice and of *miR-221* in both genders, did not follow the expression of examined mRNAs.

Summarizing our results, except for *miR-34a, miR-93* and *miR-221*, the other gene expression alterations showed a positive correlation with cell death and tumour suppression. Presumably the biodiesel glycerol, with 85% glycerol and methanol concentration less than 0.04%, could be used as a food additive safely.

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