The examination of the effect of biodiesel by-products and raw-materials on animals

PhD - Thesis

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I. Introduction

I.1. Biofuels

While the amount of fossil fuels is reducing, global energy demand is consistently increasing. Biofuels are alternative energy sources which may help to reduce air pollution, as well as our dependence on petroleum for energy. The so-called first generation biofuels refer to fuels that have been derived from food crops. The second generation biofuels, also known as advanced biofuels, are fuels that can be manufactured from various types of biomass such as the by-products of agriculture, forestry and related industry.
Sustainability criteria for biofuels and bioliquids are regulated by laws, protocols and action plans in the European Union and Hungary. In the European Union, the turn towards renewable energy sources has increased the production of biodiesel from rapeseed oil.

I.2. Biodiesel production

I.2.1. Biodiesel production from natural crops

Biodiesel can be produced from a variety of natural crops including rapeseed, soybean, mustard, flax, sunflower, canola and palm oil. There are cultivatable agricultural fields in Hungary which are suitable for growing vegetable for biodiesel.
Biodiesel is an ester based fuel for diesel engines produced by transesterification of short chain fatty acids of plant origin and mono alcohols, such as methanol. Biodiesel can be used on its own instead of fossil fuels or mixed with them. The transesterification reaction of the triglyceride from corn oil with an alcohol results in formation of esters of alcohols and glycerol. Glycerol is a natural liquid substance registered in the European Union as a feed additive, its code is E422.

I.2.2. Biodiesel production from animal fat or used cooking oil

Biodiesel can be produced from animal fat or used cooking oil as well. However, this process is more difficult and expensive than the production from natural crops.

I.3. Experiments with biodiesel glycerol form natural vegetable oils

For each litre of biodiesel, approximately 80-100 gram of crude glycerol is produced. Pure glycerol is colourless, odourless, nontoxic and it's made of 3 carbon atoms, 8 hydrogen atoms and 3 oxygen atoms. Although glycerol in its pure form is used to manufacture soap, cosmetics, pharmaceuticals and other products, the biodiesel industry is producing more of this compound than the market can cope with. This has led to renewed efforts to find new ways for the utilisation of glycerine. One proposal is to use it as a pure energy source in animal diet. Several studies have evaluated the application of glycerol in diets for poultry, pigs and ruminants, and they found no detrimental effects on growth performance or meat quality.

I.4. Experiments with biodiesel by-products or raw-materials of biodiesel

I.4.1. Biodiesel glycerol

One study, carried out at the University of West Hungary, Faculty of Agricultural and Food Sciences, investigated the metabolic effects of glycerol on boilers and barrows. Animals were fed 0, 5, 10 or 15% food grade (86,3 %) biodiesel glycerol, and they found that glycerol supplementation did not affect the quality and chemical composition of the meat, and the average daily weight did not differ between experimental and control animals.
Szendi et al and *Gerencsér et al* from the University of Pécs studied the possible genotoxic effects of oral exposure of biodiesel glycerol. In a combined in vivo/ in vitro Ames test, Long

Evans rats were feed with 2000 mg/kg biodiesel glycerol. Ames test was made from urine samples, while blood samples were taken for comet assay. They did not find any mutagenic or toxic effects of glycerol.

I.4.2. Soapy water

During purification of the crude biodiesel with slightly acidic warm water, a so-called soapy water fraction, containing glycerol, fatty acids, fatty acid methyl ester and various salts, is gained. The composition of the soapy water can provide important trace elements to the microorganisms in the soil, thus it has been suggested that it could be used to improve soil quality.
Gerencsér et al studied the ecotoxicological effect of soapy water with white mustard (Sinapis alba) root growth assay. They mixed different concentration of soapy water with soil and examined the survival of worms in this soil. Ecotoxicological effect was not found.

I.4.3. Corn oil and yellow grease

We investigated the environmental effect of used corn oil (corn oil) and used cooking oil (yellow grease), because it has been suggested using it as biodiesel raw-material.
Ames test was carried out by *Szendi et al*. Although they did not demonstrate any mutagenic activity of neither corn oil nor yellow grease, the white mustard root growth test suggested ecotoxicological effect of both. According to the results, the corn oil and the yellow grease are not suitable to improve soil quality, but their applicability as feed additive is not clear.

I.5. Molecular epidemiologic experiments with biodiesel by-products and biodiesel raw-materials

To date, no studies investigated the carcinogenic effects of the by-products of biodiesel produced from vegetable oil and corn oil or yellow grease. One aim of the molecular epidemiological studies is to detect the cell damage with early biomarkers, which can be the tool of primer prevention, because they can indicate with modified expression the changed state even before the development of invasive tumours.

I.5.1. Regulation of apoptosis

The genes which regulate signalling pathway are also considered as early biomarkers. The altered expression of these genes suggested the effect of exposure on apoptosis or cell cycle. We aimed to analyse the effects of investigated samples on the expression of three genes which play crucial role in the regulation of apoptosis and cell death: the nuclear factor kappa-light-chain-enhancer of activated B-cells 1 *(Nfkb1),* the growth arrest and DNA-damage-inducible protein 45 alpha (*Gadd45a*) and the mitogen-activated protein kinase 8 (*Mapk8, JNK1*).

I.5.2. Regulation of biotransformation

The effect of exposure can be detected in the changed biotransformation. The enzymes that play a role in the metabolism of xenobiotics can be classified into two main groups. Phase I metabolic enzymes produce small chemical changes that make a compound more hydrophilic, so it can be effectively eliminated by the kidneys. For example, Cytochrome P450 enzymes are responsible for most phase I reactions. Phase II metabolic enzymes are activated if phase I is insufficient to clear a compound from circulation, or if phase I generates a reactive metabolite. The changes in the gene expression of cytochrome P450 enzymes can be a first sign of damaging effect. We 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.

I.5.3. Oncogenes and tumour suppressor genes

A proto-oncogene is a normal gene that can become an oncogene due to mutations or increased expression. Activated oncogenes can be responsible for the survival and proliferation of the cells which are designated for apoptosis. A tumour suppressor gene, or antioncogene, is a gene that protects a cell from progressing towards the direction of cancer. The oncogenes and tumour suppressor genes are also early biomarkers, as the examined classical oncogene: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog *(K-ras).*

I.5.4. microRNAs

Recently microRNAs (miRNA) have become the focus of molecular genomic research. There is increasing interest in the relationship between miRNA expression profile and exposure to carcinogenetic agents. They can function as oncogenes or tumour suppressors, and also have a role in immune response. The expression of miRNAs is associated with disease outcomes, postsurgical recurrences and even metastatic potential in cancer patients. miRNA expression profiles can have predictive value for assessing chemical carcinogenesis.
We evaluated the expression of some tumour suppressor miRNAs *(miR-34a, miR-143, miR-146a, miR-203, miR-223),* and some oncomirs (*miR-21, miR-27a, miR-93, miR-148a, miR-155, miR-196a, miR-205, miR-221*).

II. Objectives

Testing the environmental effects of biodiesel raw materials and by-products is essential before recycling them as feed additive or using them to improve soil quality. Our aim is to examine the corn-based biodiesel by-products, the corn oil and the yellow grease, and study their effect on carcinogenesis.

II.1. The effect of biodiesel glycerol

Our objective is the investigation of environmental health risk of biodiesel glycerol (with different concentration) on apoptosis, biotransformation, *K-ras* oncogene and several miRNAs.

II.2. The effect of soapy water

Our objective is investigation of environmental health risk of wheat growing on soil treated with different concentration soapy water on apoptosis, biotransformation and several miRNAs.

II.3. The effect of corn oil and yellow grease

Our objective is the investigation of environmental health risk of corn oil and yellow grease on apoptosis, *K-ras* oncogene and several miRNAs.

III. Materials and methods

III.1. Research animals

Our research was funded by a tender and the investigations were carried out as part of a consortium. The consortium aims to produce biodiesel by-products which can be recycled safely. Our institute was responsible for carrying out the project on environmental health inspections.
Experiments were carried out according to Hungarian laws, and the Code of Ethics and Animal Research of University of Pécs.
In the experimental cancer research particular inbred rodents were used which have spontaneous incidence of tumours. A short-term animal test system has been developed in our institute, which indicated in a very early

stage of the carcinogenic effect of chemical carcinogens. Three different inbred mouse types were avaiable during the research period: CBA/Ca, BALB/c and AKR/J. Each mouse type shows elevated susceptibility for tumour formation, they are equally sensitive for various carcinogenic effects. We determined the gene expression in each kind of mice to determine the difference between the results.

III.2. Investigated samples

The biodiesel glycerol and soapy water was produced by KUKK K+F Ltd. (Budapest), the standard chew pellet by Szinbád Ltd. (Gödöllő), corn oil and yellow grease by QS Biodízel Ltd. (Newton, USA), its contains:

- standard chew pellet: energy: 11 MJ/kg, dry matters: 86%, crude protein: 20%, enzyme protein: 18,2%, lysine: 0,97%, methionine: 0,30%, cysteine: 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
- low purified biodiesel glycerol: glycerol: 60%, vegetable oil: 20%, P: 4%, Na: 1%, K: 5%, methanol: 0,04%, water and other mineral components: 9,96%
- low purified biodiesel glycerol: glycerol: 86,3%, vegetable oil: 5%, P: 2%, Na: 1%, K: 2%, methanol: 0,04%, water and other mineral components: 5%
- soapy water: methanol: 23, 3 m/m%, glycerol < 0,06 m/m %, Cl: 36,7 mg/l, P: 71,7 mg/l, S: 38,5 mg/l, nitrite: 0,3 mg/l, nitrate: 29,2 mg/l, fatty acids < 0,1 m/m%
- the composition of corn oil and yellow grease, which compounds are used as biodiesel raw materials, constantly changes.

III.3. Exposure

Mice received humane care and the experiment was carried out under the approval of the Institutional Revision Board. Mice were 6 weeks old, between 20-25 grams. Mice were fed with 10% purified biodiesel glycerol, corn oil and yellow grease. Wheat, which was growing on soil treated with different concentrations of soapy water, was given in 100% to mice. Control animals consumed standard chew pallet. The daily food intake was 3 grams. After administration, animals were sacrificed by cervical dislocation and the examined tissues of the animals were removed during autopsy. The tissues were homogenized and pooled by group.
Subsequently, 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.

III.4. Experiment groups (A1, A2, A3, B, C)

The experiments were maintained in three main groups: A, B and C, and there were three sub-group in the A main group: A1, A2, A3. The details are shown in Table I., as inbreed mouse type, exposition material and time, the investigated genes.

III.5. Mesaure of gene expression

We used the kits and instrument of Roche (Berlin, Germany) during the experiments. Total cellular RNA was isolated from the homogenized tissues with High Pure miRNA Isolation Kit according to the manufacturer's instructions. The concentration of RNA was detected by absorption photometry. Total RNA was reverse transcribed, then PCR reactions were carried out. Primers were selected by the primer finder database (www.applied-science.roche.com) and were synthesized by TIB Molbiol, ADR Logistics.
The gene expressions of mRNAs were calculated relative to the expression of *Hprt*, miRNAs expression to 5S rRNA. Statistical evaluation was carried out by paired *t*-test using STATA Release 11 software for Windows (StataCorp LP, Texas, USA). Values of $p<0.05$ were considered to be statistically significant.

III.6. Statistical probes

All PCR reactions were run in triplicates in separate runs. The concentration of miRNAs and mRNAs were determined in tissues and averaged.
Statistical evaluation was carried out by paired *t*-test using STATA Release 11 software for Windows (StataCorp LP, Texas, USA). Values of $p<0.05$ were considered to be statistically significant. Some investigator defined that miRNA expression is elevated twice or even tenfold in tumour tissue compare to normal. In order to avoid false positive results, we defined that miRNAs expression changes have to be elevated at least three times to determine significant impact in the carcinogenesis.

Table I. Experimental groups

Vizsgálati csoportok	A1	A2	A3	B	C
Mice	CBA/Ca	CBA/Ca	Balb/c	AKR/J	Balb/c
Samlpes	60% biodiesel glycerol	86,3% biodiesel glycerol	86,3% biodiesel glycerol	wheat 1: untreated,	corn oil
	86,3% biodiesel glycerol			wheat 2: 1000 l/ha,	yellow grease
				wheat 3: 500 l/ha,	
				wheat 4: 250 l/ha,	
				wheat 5: 125 l/ha soapy water	
Exposure times	3, 6 and 24 hours (h)	3, 6 and 24 hours (h)	24 hours	25 hours	26 hours
Experimental groups					
female (n)	1. control (15)	1. control (60)	1. control (30)	1. wheat 1 (10)	control: A/3
	2. 60% biodiesel glycerol, 3 h (15)	2. 86,3% biodiesel glycerol, 3 h (60)	2. 86,3% biodiesel glycerol	2. wheat 2 (10)	1. corn oil (24)
	3. 60% biodiesel glycerol, 6 h (15)	3. 86,3% biodiesel glycerol, 6 h (60)		3. wheat 3 (10)	2. yellow grease (24)
	4. 60% biodiesel glycerol, 24 h (15)	4. 86,3% biodiesel glycerol, 24 h (60)		4. wheat 4 (10)	
	5. 86,3% biodiesel glycerol, 3 h (15)			5. wheat 5 (10)	
	6. 86,3% biodiesel glycerol, 6 h (15)				
	7. 86,3% biodiesel glycerol, 24 h (15)				
male (n)	8. control (15)	5. control (60)	3. control (30)	6. wheat 1 (10)	control: A/3
	9. 60% biodiesel glycerol, 3 h (15)	6. 86,3% biodiesel glycerol, 3 h (60)	4. 86,3% biodiesel glycerol	7. wheat 2 (10)	3. corn oil (24)
	10. 60% biodiesel glycerol, 6 h (15)	7. 86,3% biodiesel glycerol, 6 h (60)		8. wheat 3 (10)	4. yellow grease (24)
	11. 60% biodiesel glycerol, 24 h (15)	8. 86,3% biodiesel glycerol, 24 h (60)		9. wheat 4 (10)	
	12. 86,3% biodiesel glycerol, 3 h (15)			10. wheat 5 (10)	
	13. 86,3% biodiesel glycerol, 6 h (15)				
	14. 86,3% biodiesel glycerol, 24 h (15)				
Examined tissues	liver, lien, bone marrow	liver	liver	liver	liver
Vizsgált gének	*Gadd45a, Nfkb1*	*Cyp1a1, Cyp2e1*	*Nfkb1, Mapk8, K-ras, miR-21, miR-27a, miR-34a, miR-93, miR-143, miR-146a, miR-148a, miR-155, miR-196a, miR-203, miR-205, miR-221*	*Nfkb1, Mapk8, Gadd45a, Cyp1a1, Cyp2e1, miR-21, miR-27a, miR-146a, miR-221, miR-223*	*Nfkb1, Mapk8, K-ras, miR-21, miR-27a, miR-34a, miR-93, miR-143, miR-146a, miR-148a, miR-155, miR-196a, miR-203, miR-205, miR-221*
Internal control	*Hprt*	*Hprt*	*Hprt,* 5S RNS	*Hprt,* 5S RNS	*Hprt,* 5S RNS

IV. Results

IV.1. Biodiesel glycerol

IV.1.1. Effect of biodiesel glycerol on apoptosis (A1 experiment)

Expressions of the investigated genes showed clearly higher gene expressions after administration of biodiesel glycerol. (Figure 1.)

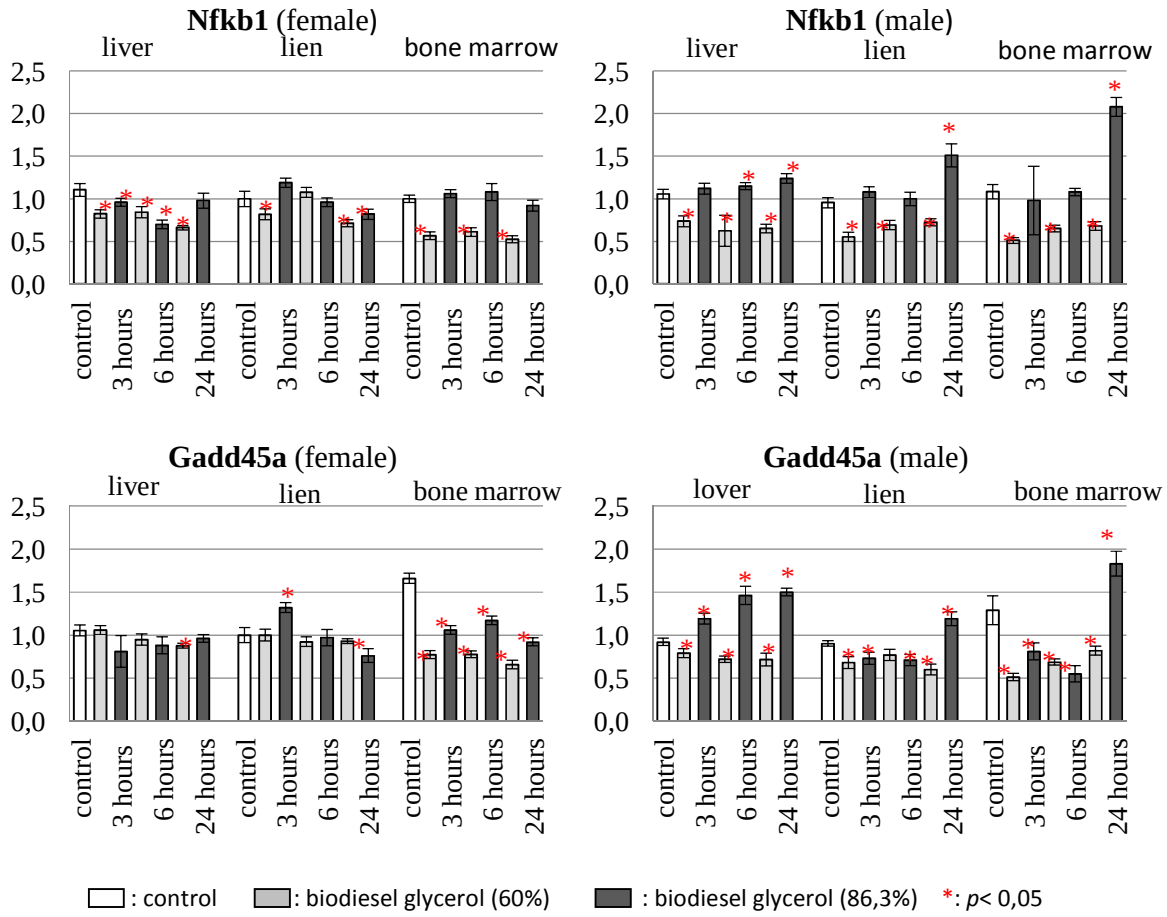

Figure 1: Gene expression of *Nfkb1* and *Gadd45a* in different tissues of female and male mice proportion to *Hprt* internal control after 3, 6 or 24 hours of biodiesel glycerol diet compared to controls

Lower purified biodiesel glycerol reduced *Nfkb1* expression nearly half of the control expression, and this downregulation of *Nfkb1* expression was seen in both males and females in every exposure times. Lower purified biodiesel glycerol consumption in male mice resulted in grater under expression than the females in all investigated organs, and the expression suppressions ranged between 47-75% of the control values at all-time points. *Gadd45a* expression showed negligible changes both in the liver and the spleen of female mice after administration. In the liver of male mice *Gadd45a* expression altered similarly to *Nfkb1* expression in all three time points.

After higher purified biodiesel glycerol administration, *Nfkb1* expression values were lower in female's liver after 3 and 6 hours of consumption, in liver after 24 hours of consumption, and *Gadd45a* expression reduced significantly in bone marrow and liver. However, in male groups *Nfkb1* expressions of the spleen and the bone marrow were higher in the 24 hour time point, surpassing the level of the control's 57% in the spleen and 82% in the bone marrow. *Gadd45a* was suppressed in the spleen and in the bone marrow of male mice after 3 and 6 hours of administration, and was elevated in liver at every time point.

Based on results obtained in the A1 experiment, we didn't continue the investigation with lower purified biodiesel glycerol, because its harmful effect could not be excluded. In the following parts of the thesis, if not marked otherwise, the biodiesel glycerol means higher purified glycerol. The change in gene expression was the most marked in the liver. Furthermore liver plays a key role in the metabolism of methanol in the body, so we continued our experiments only in liver.

IV.1.2. Effect of biodiesel glycerol on biotransformation (A2 experiment)

According to our results, the expression of *Cyp1a1* and *Cyp2e1* in liver was significantly higher at the 3hour time point in both genders. *Cyp2e1* overexpression was more marked in both sexes, reaching a four-fold overexpression in female mice and a three-fold in the males. At the 6-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 males. Figure 2. shows that the up-regulation of the genes returned to the expression levels of the controls within 24 hours from administration.

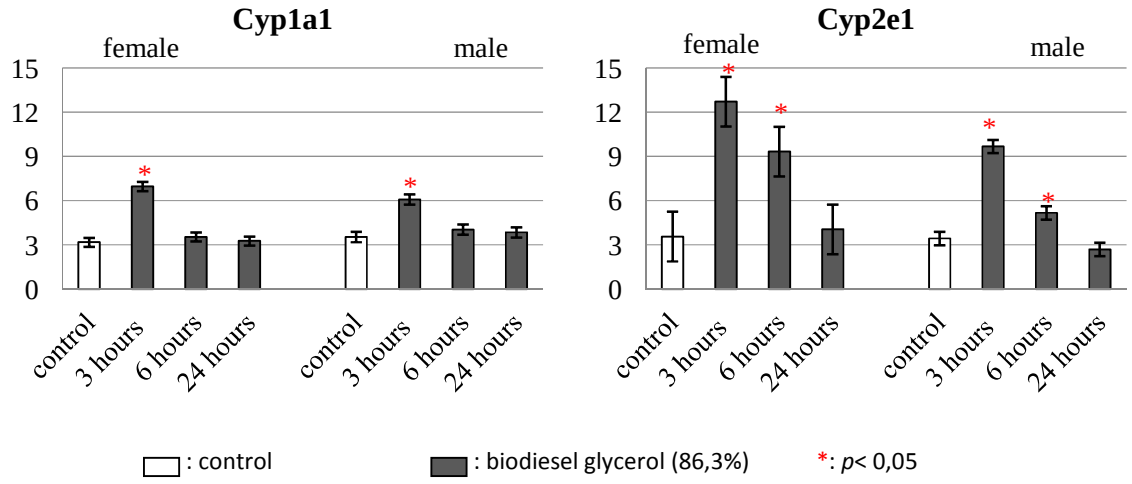

Figure 2: Gene expression of *Cyp1a1* and *Cyp2e1* in liver of female and male mice proportion to *Hprt* internal control after 3, 6 or 24 hours of biodiesel gycerol diet, compared to controls

The change in gene expression was the most marked after 24 hours administration, so we continued our experiment only with 24 hours exposure.

IV.1.3. Effect of biodiesel glycerol on K-ras oncogene, oncomirs and tumour suppressor miRNAs and mRNAs expression (A3 experiment)

Male mice exhibited more significant expression changes in the investigated miRNAs than females. In the group of female mice, the oncogene *miR-27a* was significantly deregulated parallel to 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' biodiesel glycerol administration resulted in the up-regulation of oncogene *miR-93a* and *miR-221*, while the also oncogene *miR-155* with *miR-196a* were observed to be down-regulated by the under-expression of *K-ras*. In male mice *Nfkb1* expression did not alter, while *Mapk8* was also down-regulated.

The investigated mRNA exhibited significant expression change in all examined animals and almost in all examined genes, while expression of oncogene *miR-21, miR-143, miR-148a* and *miR-205* and tumour suppressor *miR-146a* in animals was similar to control.

The expression alteration reached the three-fold level only in few cases. The results are show in Figure 3.

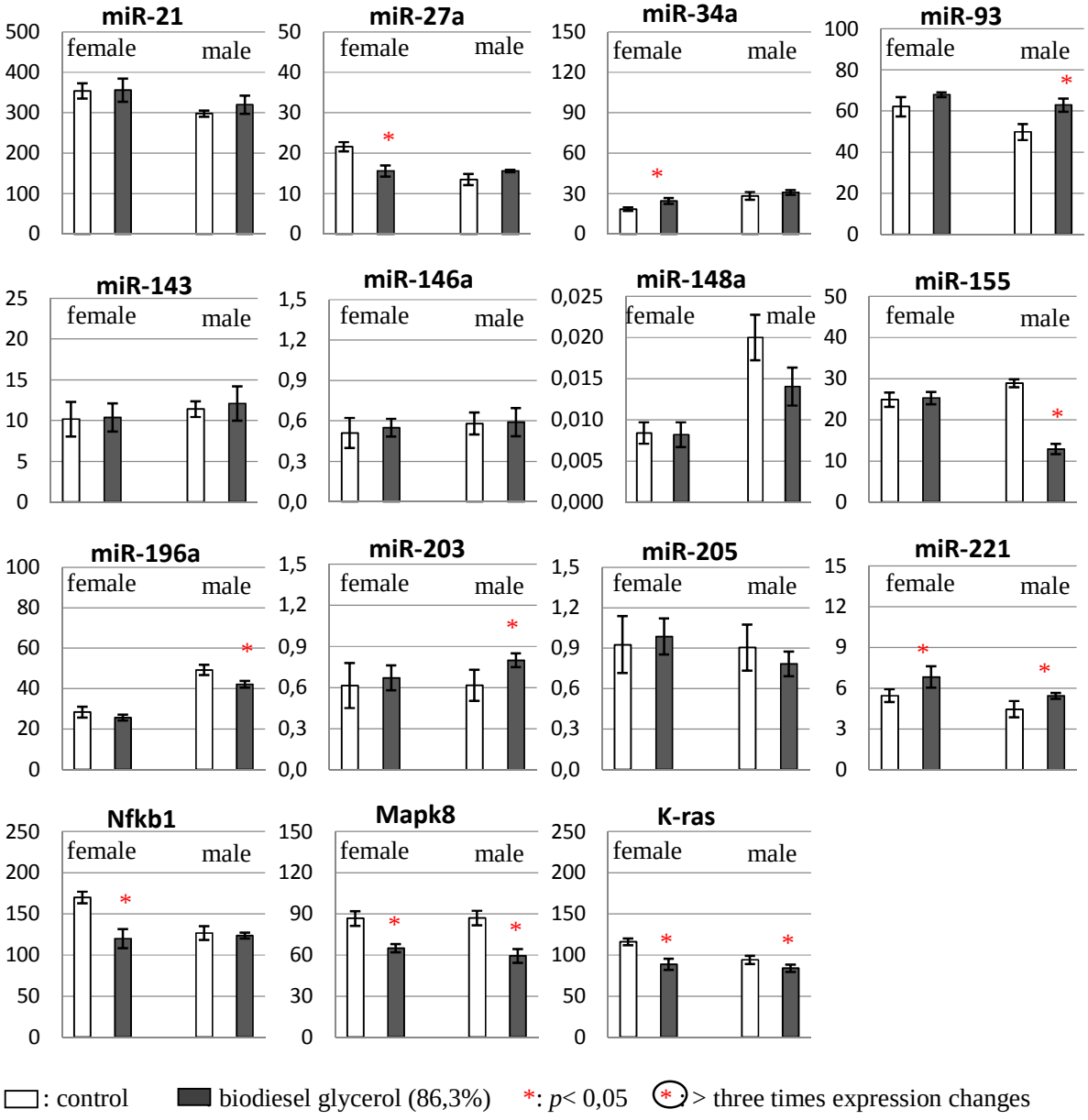

Figure 3: Gene expression of *miRNAs* compared to 5S RNA internal control, and *Nfkb1, Mapk8, K-ras* genes proportion to *Hprt* internal control in liver of female and male mice after 24 hours of biodiesel glycerol diet, compared to controls

IV.2. Effect of wheat, grown in soapy water treated soil, on apoptosis, biotransformation, oncomirs, tumour suppressor miRNAs and *K-ras* oncogene (B experiment)

We compared the effect of wheat grown in soapy water treated soil to wheat grown in normal soil.

Wheat grown in soil treated with 1000 l/ha soapy water showed significantly overexpression of a *miR-21* and significantly downregulation of *miR-146a* in male mice, but these changes

didn't reach the 3 time level. The level of *miR-221* was more than three times higher in every gender compared to controls.
Wheat grown in soil treated with 500 l/ha soapy water supressed the expression of *miR-221* with 60%.
There were no significant changes in the expression of miRNAs after administration of wheat grown in soil treated with 250 l/ha soapy water.
Nfkb1 expression was significantly higher, while *Mapk8* was significantly lower after 1000 l/ha and 500 l/ha soapy water treatment in both gender, and *Nfkb1* was also overexpressed in female mice in the case of 250 l/ha concentration.
The soapy water had effect on biotransformation only in 1000 l/ha concentration. *Cyp1a1* was under expressed in female and *Cyp2e1* in male mice. (Figure 4.)

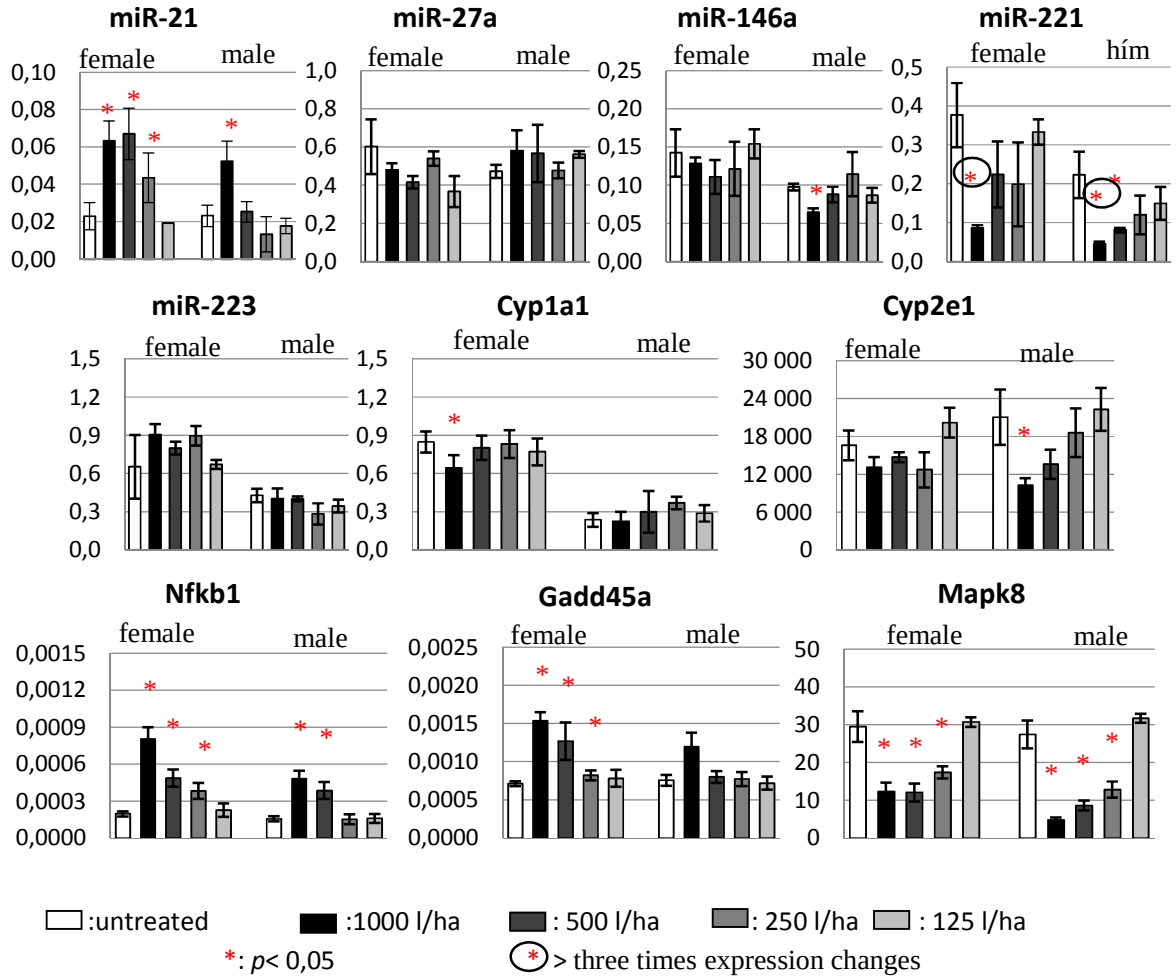

Figure 4: Gene expression of *miRNAs* compared to 5S RNA internal control, and *Nfkb1, Gadd45a, Mapk8, Cyp1a1, Cyp2e1* genes proportion to *Hprt* internal control in liver of female and male mice after 24 hours diet with wheat growing on soil treated with soapy water in different concentration, compared to controls

IV.3. Effect of corn oil and yellow grease on apoptosis, on oncomirs and tumour suppressor miRNAs and *K-ras* oncogene (C experiment)

Expression of the investigated genes showed many difference between controls and mice consumed corn oil or yellow grease. (Figure 5.)
Corn oil administration reduced the expression of *miR-21*, *miR-27a*, *miR-93*, *miR-143*, *miR-146a*, *miR-155*, *miR-203* and *miR-221* level in female mice, reduced one-third of the *miR-27a*

and the *miR-146a.* In male liver, suppressed expression of *miR-34a*, *miR-93*, *miR-143*, *miR-146a*, *miR-155*, *miR-196a*, *miR-203* and *miR-221 were showed.* From these, only the *miR-146a was* reduced by third, and the *miR-27a* was overexpressed. The *Mapk8* expression was lower in female mice, while *Nfkb1* and *K-ras* in both genders compared to controls.
After administration of yellow grease, *miR-21*, *miR-27a*, *miR-34a*, *miR-93*, *miR-155* and *miR-221* were downregulated in both genders. The *miR-143*, *miR-146a* and the *miR-205* were suppressed in females, *miR-148a* and *miR-196a* in males. Two miRNAs were upregulated in male mice: *miR-146a* and *miR-203. Nfkb1* and *K-ras* was overexpressed in males.

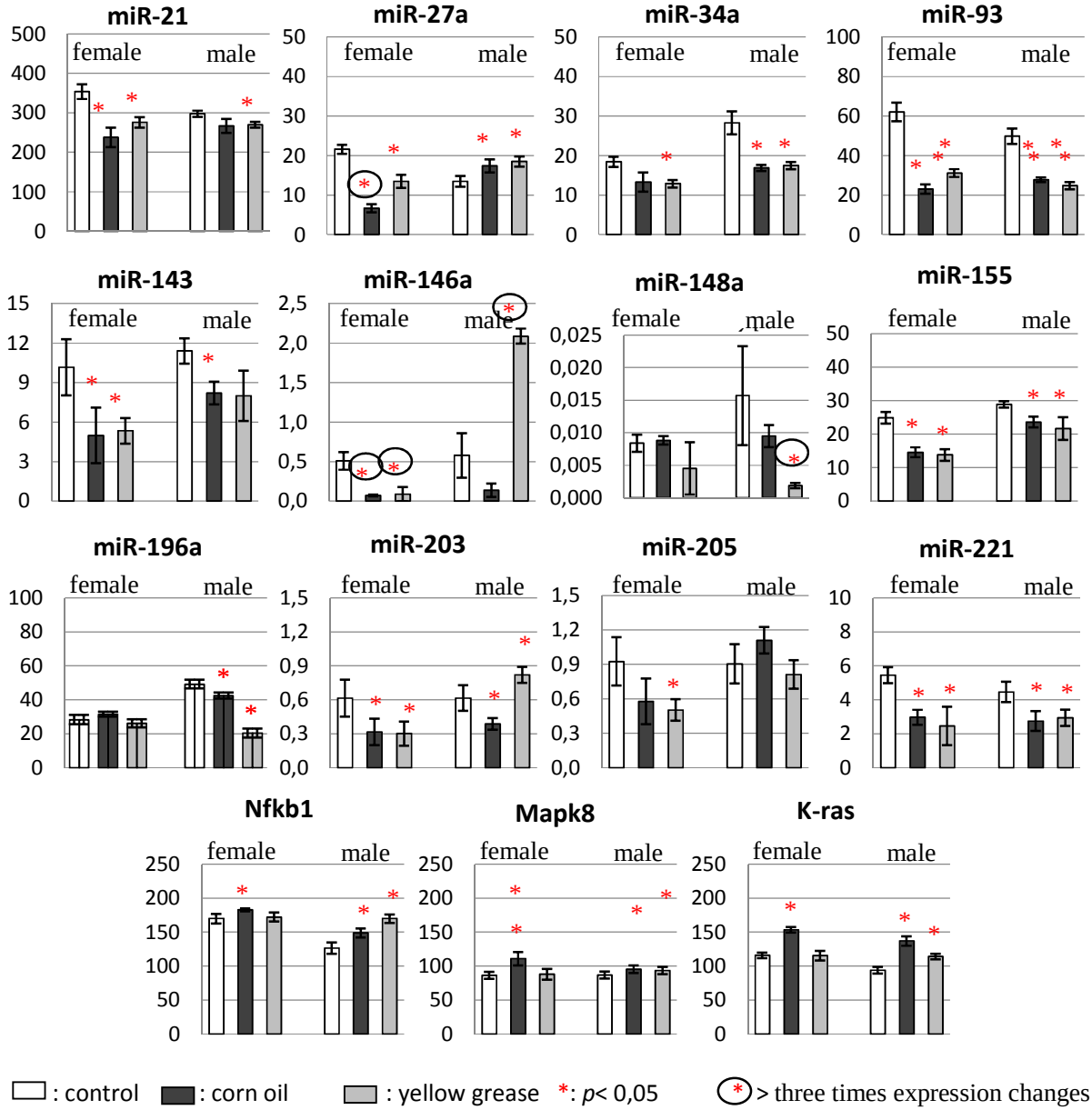

Figure 5: Gene expression of *miRNAs* compared to 5S RNA internal control, and *Nfkb1, Mapk8, K-ras* genes proportion to *Hprt* internal control in liver of female and male mice after 24 hours diet with corn oil or yellow grease, compared to controls

V. Discussion

The bioutilization and biological effect of biodiesel by-products *in vivo* strongly depend on the impact of trace elements, saturated and unsaturated fatty acid and methanol content of the product. Our aim was to develop the technology for biodiesel glycerol purification, in order to gain a glycerol fraction that could be physiologically optimal for animal feeding, with the cost effectively highest purity and with the lowest possible risk of carcinogenicity. It would be more cost-effective and environmental friendly to use the by-product of a renewable energy product.

The corn oil and yellow grease can be used for biodiesel production, and we investigated whether they are suitable as food additive too.

V.1. Regulation of apoptosis

NFkB 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 NFkB family includes homo- and heterodimers that are formed from five structure-related protein subunits: p50 (NFkB1), p52 (NFkB2), p65 (RelA), c-Rel, Rel B. NFkB can be found in various cells. NFkB normally binds to IkB inhibitors. Stress stimulates IkB kinase, and activated IkB kinase mediates IkB phosphorylation. The NFkB-IkB complex degrades and the free NFkB translocates into the nucleus and regulates the transcription of target genes. NFkB 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.

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* leads to NfkB-dependent escape from programmed cell death through the JNK cascade.

The arsenite-induced AP-1 activation was delivered by sequential induction of GADD45a expression and the activation of MAPKK (MKK3/4/6) and MAPK (JNKs and p38K)-dependent pathways.

V.1.1. Effect of biodiesel glycerol on apoptosis

Administration of lower purified biodiesel glycerol caused downregulation of *Nfkb1* and *Gadd45a* in almost every tissue, in every time point and in both genders. This downregulations reached the remarkable 50% in the bone marrow of both sexes. Based upon this finding, we can conclude that implication of biodiesel glycerol in the diet results in early gene expression downregulation on the extrinsic apoptotic cascade *via* suppression on the DNA damage inducible *Gadd45a* expression and suppression of *Nfkb1*, potentiating cell survival mechanisms of the cells with damaged genetic material. Therefore, our workgroup though that it is not safe to use it as a feed composition.

Higher purified biodiesel glycerol had much smaller effect on gene expressions of the vast of the investigated organs, although isolated induction of *Gadd45a* were seen in the spleen and bone marrow of male mice in the 24 hour time point and in liver in every time point. *Nfkb1* expression was lower in female BALB/c mice, and higher in male CBA/Ca mice compared to the control, and the *Mapk8* was downregulated in BALB/c mice. By the alteration of Nfkb1 and Mapk8 gene, it can be apoptotic or antiapoptotic too, but the upregulated Gadd45a even suggested antiapoptotic effect.

These data suggest that glycerol fractions with higher purity has no harmful effect on the apoptotic signalling, thus on the evasion of apoptosis and cell survival.

V.1.2. Effect of wheat growing on soil treated with soapy water on apoptosis

Wheat growing on soil treated with soapy water caused downregulated *Mapk8* and upregultaed *Nfkb1* levels, and also the expression was elevated of *Gadd45a*, but it was only significant in female mice. According to *Gadd45a* upregulation and the inverse expression of *Nfkb1* and *Mapk8*, it seems to have apoptotic effect. Our result suggests that soapy water has no harmful effect on apoptosis.

V.1.3. Effect of corn oil and yellow grease on apoptosis

According to our result with corn oil, the expression of *Nfkb1* was elevated in both gender, and *Mapk8* was upregulated in female liver. The yellow grease had effect only on *Nfkb1* gene expression, which was elevated in male.

Both corn oil and yellow grease have effect on investigated genes in males, but yellow grease has effect only on males. Our results suggest that these materials influence the expression of genes, which play central role in cell survive.

V.2. Regulation of apoptosis

Cyp1a1 encodes a protein which is localised in the endoplasmic reticulum. Endogenous substrates of *Cyp1a1* are steroids and fatty acids, and it also partakes in metabolism of caffeine, pethidine, phenacetin, progesterone and other steroids and polycyclic aromatic hydrocarbons. *Cyp2e1 is also* localised in the endoplasmic reticulum, and it is involved in the 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. In addition, in rodents, alcohol dehydrogenisation is also maintained mostly by *Cyp2e1* and less by the alcohol dehydrogenases. 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, it produces powerful oxidants such as the hydroxyl radical. *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.

V.2.1. Effect of biodiesel glycerol on biotransformation

According to our results, the expressions of both *Cyp1a1* and the *Cyp2e*1 were elevated after supplementing the animals' diet with biodiesel glycerol, as a response to the exposure due to its variety of lipid contaminants and methanol. This upregulation quickly subsided and expressions equilibrated with those of the controls within six hours, suggesting that the pathway of biotransformation adapted to the metabolic needs. No significant alterations of the genes were seen after 6 and 24 hours of exposure. 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-oxygenize activity is modified by sex hormones.

Based upon our data, the higher purified biodiesel glycerol 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.

V.2.2. Effect of wheat growing on soil treated with soapy water on biotransformation

The wheat which was grown in soil treated with 1000 l/ha soapy water had effect only on cytochromes. The expression of *Cyp1a1* was decreased in female mice, while the expression of *Cyp2e1* was lower in the liver of male mice. The lower concentration of soapy water had no effect on biotransformation, but it is possible, that methanol can cause changes in 1000

l/ha concentration, so we suggest to avoid using it for soil quality improvement in this concentration.

V.3. Effects on *K-ras* oncogene

Rat sarcoma virus oncogene (RAS) proteins were originally identified as retroviral oncogenes, they are small GTPases. RAS proteins control cellular signalling pathways responsible for growth, migration, adhesion, cytoskeletal integrity, survival and differentiation. As many human tumours have activating mutations in one of the *RAS* genes, RAS signalling may result in malignant transformation.

V.3.1. Effects of biodiesel glycerol on K-ras oncogene

After exposure with higher purified biodiesel glycerol, the expression of *K-ras* was lower than in controls, therefore biodiesel glycerol seems to be a protective agent.

V.3.2. Effects of corn oil and yellow grease on K-ras oncogene

Administration of corn oil elevated *K-ras* expression in both genders. It was overexpressed in male's liver after diet with yellow grease.
Our results suggest that corn oil and yellow grease has carcinogenetic effect, therefore they should not be used as feed additive or for soil quality improvement.

V.4. Effect on oncomirs and tumour suppressor miRNAs

Several studies have shown that miRNAs regulate cell proliferation and apoptosis, they 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.
The miRNA profile can be different in alcoholic and non-alcoholic steatosis. The liver contains many types of cells, including parenchymal cells (i.e. hepatocytes) and "non-parenchymal cells" which can be endothelial cells, stellate cells, lymphoid cells, and biliary epithelial cells (cholangiocytes). Each cell type may have completely distinct miRNA expression profile because most of the cells are hepatocytes and the homogenized tissue expression profile is typifying hepatocytes.
The oncomirs are the follows: *miR-21, miR-27a, miR-93, miR-148a, miR-155, miR-196a, miR-205, miR-221*. *miR-21* has multiple functions: it promotes cell proliferation, migration, survival and also promotes cyclin D1 translation; it helps hepatocytes to exit from the G0 phase of the cell cycle to progress through the G1 phase; it also inhibits the negative regulators of the Ras/MEK/ERK pathway and inhibit apoptosis.
The increased expression of the *miR-23a-27a-24* cluster decreased the level of the tumour suppressor transforming growth factor-beta (*Tgf-b*) in hepatocellular carcinomas. *miR-27a* may modulate expression of *miR-27a* a zinc finger and BTB domain containing 10 (*Zbtb10*) and myelin transcription factor 1 (*Myt-1*) tumour suppressor genes, that inhibit cells from progressing past G_2-M phase. *miR-93* promotes tumour growth and angiogenesis by suppressing integrin-β8 expression and inhibited Fus1 protein expression. Elevated expression of *miR-148a* can be detected in aggressive tumour tissues, it inactivates the tumour suppressor phosphatase and tensin homolog (PTEN). 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 via animal diet causes hepatocellular carcinoma. *miR-196a* promoted the epithelial-mesenchymal transition and migration/invasion capabilities of transfected cells, suggesting its oncogenic potential, and promotes cell proliferation by downregulating p27. *miRNA-221* in tumour samples target the CDK inhibitor p27 and enhance cell growth in vitro, and *miR-221* overexpression stimulates growth of tumourigenic murine hepatic progenitor cells.

The tumour suppressors are: *miR-34a, miR-143, miR-146a, miR-203* and *miR-223*.
The *miR-34a,* as a tumour suppressor, suppresses tumour migration and invasion through modulation of the c-Met signalling pathway. KRAS oncogene has been further experimentally validated as the target of *miR-143*, there is an inverse correlation between KRAS protein and *miR-143.* Expression of *miR-146a* suppresses NF-kB activity and reduces the metastatic potential of tumour cells. Functional polymorphism in the *miR-146a* gene is associated with the risk for hepatocellular carcinoma. *miR-203* was found to be tumour suppressor miRNA in hepatocellular carcinoma. The ATP-binding cassette, sub-family E (OABP), member 1 (*ABCE1)* is a possible target for *miR-203.* ABCE1 is known to bind with eukaryotic initiation factors and play a role in vertebrate translation initiation. ABCE1 inhibitors were shown to efficiently suppress the growth of human tumour cells. One of the targets of *miR-205* is the *K-ras* oncogene. In hepatocellular carcinoma cells *miR-223* was repressed compared with normal liver tissue. It seems to inhibit insulin-like growth factor-1 receptor (IGF-1R) signal pathway, as a tumour supressor gene.

V.4.1. Effect of biodiesel glycerol on miRNAs expression

Biodiesel glycerol diet had no remarkable effect on the expression of miRNAs. The elevation of their expression did not reach the three-fold level and their quantity did not decrease to its third..

V.4.2. Effect of wheat grown in soapy water treated soil on miRNAs expression

Consumption of wheat grown on soil treated with 1000 l/ha soapy water dowregulated the expression of *miR-221* in both genders by one- third, but it had no more remarkable effect.

V.4.3. Effect of corn oil and yellow grease on miRNAs expression

The level of oncomir *miR-27a* in females, and the level of tumour suppressor *miR-146a* in males decreased to its third after corn oil diet.
After administration of yellow grease, the tumour suppressor *miR-146a* decreased more than a third in female mice's liver while in male mice increased more than fourfold. The expression of oncogenic *miR-148a* decreased to its eighth in males.
Carcinogenic effect was not confirmed on the basis of differences in miRNA expression in either corn oil or consumption of yellow grease enriched diet.

VI. Summary and the new findings

Biofuel companies can produce biodiesel in principally from vegetable oil, but due to the economic requirements they try to produce it from used cooking oil and animal fats. The rising production of rapeseed evoked the necessity of the utilization of the valuable by-products, the glycerol fractions.
At the Institute of Public Health at the University of Pécs we examined the alteration in gene expression profiles in animals after administration of biodiesel by-products and raw materials. We studied their effect on carcinogenesis and found that they could be physiologically optimal for animal feeding, or for using them as soil quality improving compounds, before they enter into the food chain.

VI.1. New findings

- According to the expression changes of examined genes, the lower purified biodiesel glycerol, may have antiapoptotic effect.
- According to the expression changes of examined genes, the higher purified biodiesel glycerol has no effect on apoptosis and has a transient effect on biotransformation. As it

suppressed the expression of oncogene *K-ras*, it can also be a protective agent. It has no remarkable effect on the examined miRNAs.

- According to the expression changes of examined genes, the wheat which was grown in soil treated with 1000 l/ha soapy water has apoptotic and metabolic effect, and can be protective agents as well, as it suppressed the expression of oncogene.
- According to the expression changes of examined genes, the wheat which was grown in soil treated with 500 l/ha soapy water, has apoptotic effect, has no effect on biotransformation and on examined miRNAs.
- According to the expression changes of examined genes, the wheat grown on soil treated with 250 l/ha soapy water, has apoptotic effect, has no effect on biotransformation and on examined miRNAs.
- According to the expression changes of examined genes, the wheat grown on soil treated with 125 l/ha soapy water has no effect on apoptosis, on biotransformation and on examined miRNAs.
- According to the expression changes of examined genes, corn oil has apoptotic effect, can be carcinogen, because it elevated the expression of *K-ras* oncogene, and has different effect on examined miRNAs.
- According to the expression changes of examined genes, yellow grease has apoptotic effect in female mice, and has no apoptotic effect in males. It can be carcinogen because it elevated the expression of *K-ras* oncogene. It also has different effect on examined miRNAs.

Based upon our data, the higher purified biodiesel glycerol can be used as feed additive and the soapy water which is less than 500 l/ha concentration is a suitable compound to improve soil quality. They pose no risk for the environment.
The utilisation of corn oil and yellow grease as raw material for biodiesel production increases the cost-effectiveness of this process, because their recycling into the food chain is not safe as long as they have carcinogenic effect.

VII. Publications

The thesis is based on the following international publications

1. Szele E, Gombos K, Kovács A, Ember I: Feeding purificated glycerol from biodiesel to CBA/CA mice: effects on Gadd45α and Nfκb1 expressions. In Vivo *24(3):* 303-307, 2010. **imp.f.: 1,159**
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1. Szele E, Gombos K, Ember I: Biodízel előállítás során a glicerin-fázis melléktermékek állati takarmánykompozícióként való alkalmazásának vizsgálata – Az SZME2 hatása az NFKB1 és GADD45α expresszióra. Magyar Epidemiológia *6(1):* 21-26, 2009.
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