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The Effect of Biofuels on Colony Formation of CHO-9 Cells

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1. Introduction

The biofuels are an interesting alternative by being a cleaner source of fuel energy. In Europe the requirement of 10% bio-component content in fuels (Directive 2015/1513/EU) will come into force until 2020. A review of literature showed that there are few toxicological, cytotoxicity and mutagenicity studies on the effects of biofuels in biological systems. According to Cavalcante et al. (2014) the cytotoxicity of water solution of biofuels is presumably related with soluble compounds originating from biofuels, such as dispersed droplets of fatty acid esters, alcohol residues (methanol or ethanol, depending on the production route), or due to the presence of elements from the raw material, the production process, or substances formed during the storage of biofuels. Identification of the composition of 75% solution of tested biofuels in a medium was carried out using gas chromatography techniques coupled with mass spectrometric detection. In the biofuel BP II from waste animals fats the highest concentrations of fatty acid esters were determined (unpublished data). Based on the results of the IC50 levels, among the analysed biofuels, the one produced from frying vegetable oils (BP III) was the most toxic on the human epithelial skin cell line A431, followed by those produced from waste animals fats (BP II), rapeseed oil (BP IV) and rapeseed expired oil (BP I) in both test: NRU – a test used to assess cell membranes integrity and MTT – a test which determines the metabolic activ-

ity of mitochondria. The higher cytotoxicity of biofuel III may be due to the contamination present in the raw material (Skowroń et al. 2015).

The clonogenic assay has been used in a variety of studies with many different cell types to detect cells that retained the capacity for producing a large number of progeny after treatments or that can cause reproductive death as a result of damage to chromosomes, apoptosis, etc. (Brown, Attardi 2005). The Chinese hamster ovary cells (CHO-9) are regarded as an appropriate model for investigating the cytotoxic effect of xenobiotics and were more sensitivity for studying these effect of cadmium compounds than human lung cancer cells A549 (Zapór 2014). In this study, the clonogenic assay is used to assess the effects of exposure to four biofuels on colony formation of CHO-9 cells.

2. Materials and methods

2.1. Cell cultures and treatment

The line of Chinese hamster ovary cells CHO-9 was kindly provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland. The CHO-9 cells were cultured as a monolayer in F-10 (Ham) medium supplemented with 7% FBS and 1% antibiotic-antimycotic (1 ml/100 ml medium) in sterile tissue culture flasks (Nunc, USA) and maintained at 37°C under the humidified atmosphere of 95% air and 5% CO_2 (pH 7.2-7.4). The cells were subcultured twice a week. Before the experiment, cell suspension was prepared and cell viability was assessed with the trypan blue exclusion assay (Tennat 1964).

2.2. Preparation of tested biofuels

Tested biofuels: I, II and III have been produced in the laboratory, based on generally used by agricultural producers of technology for the production of biofuels, low-temperature transesterification (Frąckowiak 2002). Biofuel IV was prepared in a factory producing biofuels from crude rapeseed oil. The tested biofuels were insoluble in water. According to the method described by Chou (2003), for the aromatic hydrocarbons which are components of aviation fuels and therefore insoluble in water, the 15 ml of tested biofuels were shaken with 5 ml culture medium in sterile flasks of 50 ml for 18 h at room temperature with a rotational

speed of 400 rpm. Then, after centrifugation, a layer of the resulting emulsion was drained and about 4 ml of the clear medium was obtained. It was assumed that it would be a solution of saturated fatty acid esters. The media was then further serially diluted, assuming 75% solution of the test biofuel in the medium as 100% in order to obtain a range of concentrations to determine the concentration-effect. The highest concentrations of the test solutions of the biofuels were strongly cytotoxic to CHO-9 cells that did not form colonies (Table 1).

Table 1. The range of concentrations of tested biofuels in media used in clonogenic assay on Chinese hamster ovary CHO-9 cells in the preliminary study
Tabela 1. Zakres stężeń badanych biopaliw w mediach komórkowych stosowanych w teście tworzenia kolonii na komórkach jajnika chomika chińskiego CHO-9 (badania wstępne)

	Tested biofuel			
The clonogenic assay	Biofuel I from rapeseed expired oil Concentration [%]	Biofuel II from waste animals fats Concentration [%]	Biofuel III from frying vegetable oils Concentration [%]	Biofuel IV from rapeseed oil Concentration [%]
Chinese hamster ovary cells CHO-9	0.188-3	0.188-0.75	0.188-1.5	0.375-6

2.3. Clonogenic assay

The clonogenic assay, also called colony formation assay or colony forming efficiency assay, is based on the ability of a single cell to grow into a colony. The procedure for the clonogenic assay was adopted from Puck and Markus (1956) and Franken et al. (2006) and cells were treated after plating. A colony being defined to consist as at least 50 clones of one cell (which corresponds to 6 mitotic divisions). Exponentially growing cells were harvested and seeded in sterile Petri dish 60 x 15 mm (21 cm^2) (Iwaki Cell Biology, Japan) at a density of

500 cells/dish together with tested biofuels. Each dish finally contained 5 ml of cell culture medium with biofuels in appropriate concentrations at least in three replicates for each treatment. Cells were exposed to biofuels over the time period they needed to form colonies, that is 7 days. After this period, particle solutions were removed, cells were washed with phosphate buffered saline (PBS), fixed (ethanol), stained (0.4% Giemza), and colonies were counted. The average plating efficiencies of cells were 56%. To calculate the survival fraction we used a formula, described as follows (Nikzad, Hashemi 2014):

$$PE = (\text{the number of colonies counted/the number of cells}) \cdot 100 \quad (1)$$

where, PE is the plate efficiency.

The surviving fraction (SF) is determined by dividing the PE of the treated cells by the PE of the controls, and then multiplying by 100:

$$SF = (PE \text{ of treated sample}/PE \text{ of control}) \cdot 100 \quad (2)$$

2.4. Statistics

At least three independent experiments were conducted for biofuels. Test results were expressed as percentage of the unexposed control ± standard deviation (SD). Control values were set as 100%. Differences between samples and the control were evaluated using t-test. Results were considered statistically significantly different at $p < 0.05$.

3. Results and discussion

As shown in Fig. 1, after a 7-day exposure of CHO-9 cells to biofuel I, colony numbers were reduced in all tested concentration ($p < 0.05$) to around 9.75%, 7.9%, 3.2% and 27.7% compared to control, respectively. The exposure of CHO-9 cells to biofuel II from animal fats for 7 days in the same concentration as biofuel I, resulted in the colony numbers reduced by: 13.5%; 21%; 82.7% and 81.7% respectively, compared to control. Biofuels III reduced the formation of CHO-9 colonies in all concentrations, but significant differences existed for the following concentrations: 0.188%; 0.375%; 0.75% and 1.5%. The colony numbers were reduced by: 3.7%; 13%; 11.2% and 97%, respectively. For a 1% solution of biofuel III, the number of CHO-9 colonies created was reduced by

51.8% relative to control but not statistically significant (large differences in the t-Student test, high SD value). For a 0.5% concentration of BP III, the ability to form colonies by CHO-9 cells was not assessed, because at a lower concentration, i.e. 0.375% and higher, i.e. 0.75%, the colony-forming capacity was at the same level. Biofuel IV derived from crude rapeseed oil reduced the colony number of CHO-9 cell at higher concentrations than other tested biofuels. At a concentration of 3% the result was statistically insignificant (large differences in the t-Student test, high SD value). At a concentration of 0.375%, a statistically significant increase in the ability of CHO-9 cells to colony formation was observed. It can be explained by the formation by some cells of large colonies that survived the exposure and maintained the ability to multiply indefinitely (Munshi et al. 2005). This can also be explained by the phenomenon of hormesis (Calabrese & Baldwin 2001). Mild to moderate exposure shifts cells to an adaptive state because the homeostatic control is operating. Higher exposure moves cells to a stressed state because the limit of homeostatic control is reached. Cells in adaptive or stressed state can still return to normal, unstressed state after removal of the stressor. However, very high doses of stressors are likely to drive cells irreversibly to a toxic state, where apoptosis or necrosis occurs (Zhang et al. 2008). At the highest concentration of biofuel IV, i.e. 6% CHO-9 cells did not form colonies.

Colony test results showed that the ability of CHO-9 cells to form colonies and the degree of inhibition were depended on the type and concentration of the biofuel. The strongest action in reducing CHO-9 cell proliferation capacity was demonstrated by biofuel II in solutions of 0.5% or 0.75% and biofuel III in 1.5% solution. Biofuel IV impacted on the ability of the CHO-9 cells to colony forming in higher concentration than others.

The ability of cells to form colonies has been widely used to screen xenobiotics for potential toxic activity (Franken et al. 2006). Only mitotically viable cells are capable of producing clones and therefore colonies. As a result, the number of colonies formed after or during treatment indicates cell viability as these cells are able to stay attached to the culture vessel surface and are able to undergo a certain amount of cell divisions. The clonogenic assay offers the possibility of long-term toxicity assessment at a sub-lethal level.

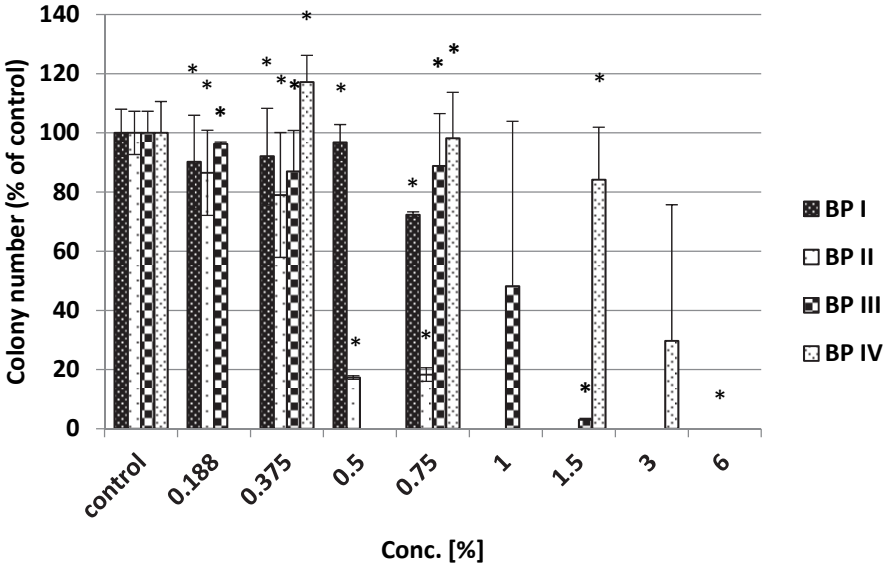

Fig. 1. Effects of biofuels exposure to colony formation of Chinese hamster ovary cells (CHO-9) cells. Bars showing colony number as determined following 7 days exposures to biofuel obtained of: rapeseed expired oil (BP I), waste animal fats (BP II), frying vegetable oils (BP III), and rapeseed oil (BP IV). Results are expressed as percent of control mean ± SD of three independent experiments. Asterisk (*) denotes a significant difference from the control ($p \geq 0.05$)

Rys. 1. Wpływ badanych biopaliw na zdolność tworzenia kolonii przez komórki jajnika chomika chińskiego CHO-9. Każdy punkt reprezentuje średnią i odchylenie standardowe (± SD) z 3 niezależnych eksperymentów po 7 dniowym narażeniu na: przeterminowany olej rzepakowy (BP I), tłuszcz zwierzęcy (BP II), posmażalniczy olej roślinny (BP III) oraz surowy olej rzepakowy (BP IV). Gwiazdka (*) oznacza różnice statystycznie istotne w stosunku do komórek nienarażanych na badane biopaliwa ($p < 0,05$)

In this study, it was possible to show differences in cytotoxicity between four types of biofuels after 7 days of exposure. Biofuel II from animal fats, which has the highest concentrations of fatty acid esters, was more reactive compared to others. Biofuel III produced from frying vegetable oils was the most toxic on A431 skin cells and the study also showed that it was more toxic to proliferation of CHO-9 cells than biofuel I or IV. The preliminary report does not provide any explanation for

this effect, but the result may nevertheless be interesting for future studies on genotoxicity of biofuels.

Bünger et al. (2007) demonstrates very strong mutagenicity of Diesel engine particles extracts and condensates from combustion of rapeseed oil and preheated rapeseed oil in the *Salmonella typhimurium*/mammalian microsome assay. The authors conclude that the strong increase of mutagenicity using rapeseed oil as diesel fuel compared to the reference Diesel fuel and other fuels causes deep concern for future usage of this biologic resource as a replacement of the established diesel fuels.

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References

Brown, J.M., Attardi, L.D. (2005). The role of apoptosis in cancer development and treatment response. *Nat. Rev. Cancer*, *5*, 231-237.

Bünger, J., Krahl, J., Munack, A., Ruschel, Y., Schröder, O., Emmert, B., Westphal, G., Müller, M., Hallier, E., Brüning, T. (2007). Strong mutagenic effects of diesel engine emissions using vegetable oil as fuel. *Arch Toxicol.*, *81(8)*, 599-603.

Calabrese, E.J., & Baldwin, L.A. (2001). Hormesis: a generalizable and unifying hypothesis. *Crit. Rev. Toxicol.*, *31*(*4/5)*, 353-425.

Cavalcante, D.G.S.M., da Silva, N.D.G., Marcarini, J.C., Mantovani, M.S., Marin-Morales, M.A., Martinez, C.B.R. (2014). Cytotoxic, biochemical and genotoxic effects of biodiesel produced by different routes on ZFL cell line. *Toxicology in Vitro*, *28(6)*, 1117-1125.

Chou, C.C., Riviere, J. E., Monteiro-Riviere, N.A. (2003). The cytotoxicity of jet fuel aromatic hydrocarbons and dose-related interleukin-8 release from human epidermal keratinocytes. *Arch Toxicol.*, *77*, 384-391.

Directive (EU) 2015/1513 of the European Parliament and of the Council of 9 September 2015 amending Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources (OJ L 239, 15.9.2015, p. 1).

Franken, N., Rodermond, H. M., Stap, J., Haverman, J., van Bree, Ch. (2006). Clonogenic assay of cells in vitro. *Nature Protocols, 1(5),* 2315-2319.

Frąckowiak, P. (2002) Testing of estrification process of rape oil for tractor fuel in the prototype production plants of 400 dm^3 output (Badania procesu estryfikacji oleju rzepakowego na paliwo ciągnikowe w prototypowych wytwórniach o wydajności 400 litrów na dobę). *Journal of Research and Applications In Agricultural Engineering*, *47(1)*, 67-73.

Nikzad, S., Hashemi, B. (2014). MTT assay instead of the clonogenic assay in measuring the response of cells to ionizing radiation. *Journal of Radiobiology, 1(1,3-8)*, doi: 10.15171/jrb.2014.02 http://www.jradiobiol.com.

Munshi, A., Hobbs, M., Meyn, R.E. (2005) Clonogenic cell survival assay From: *Methods in Molecular Medicine, vol. 110: Chemosensitivity: Vol. 1: In Vitro Assays* Edited by: R. D. Blumenthal © Humana Press Inc., Totowa, NJ, 2005. http://www.springer.com/978-1-58829-345-9.

Puck, T.T., Markus, P.I. (1956) Action of X-rays on mammalian cells. *J. Exp. Med., 103*, 653-666.

Skowroń, J., Zapór, L., Miranowicz-Dzierżawska, K. (2015). Cytotoxicity of biofuels produced by esterification of waste materials: vegetable oils or animal fats on A431 skin cells. *Toxicology Letters*, *238(2S), 345*. http://dx.doi.org/10.1016/j.toxlet.2015.08.985.

Tennat, J. A. (1964). Evaluation of trypan blue technique for determination of cell viability. *Transplantation*, *285-692*.

Zapór, L. (2014). Evaluation of the Toxic Potency of Selected Cadmium Compounds on A549 and CHO-9 Cells. *International Journal of Occupational Safety and Ergonomics*, *20(4), 573-581*, doi: 10.1080/10803548.2014.11077080.

Zhang, Q., Pi, J., Woods, C.G., Jarabek, A.M., Clewell, H.J., Andresen, M.E. (2008) Hormesis and adaptive cellular control systems. *Dose-Response, 6, 196-208*. doi: 10.2203/dose-response.07-028.Zhang

Wpływ biopaliw na tworzenie kolonii przez komórki CHO-9

Streszczenie

Oceniano wpływ czterech biopaliw otrzymanych w reakcji transestryfikacji tłuszczów odpadowych: przeterminowanego oleju rzepakowego (BP I), tłuszczu zwierzęcego (BP II), posmażalniczego oleju roślinnego (BP III) oraz surowego oleju rzepakowego (BP IV) na zdolność komórek jajnika chomika chińskiego (CHO-9) do procesów odnowy i prawidłowego namnażania się (test klonogenny). Badane biopaliwa hamowały zdolność komórek jajnika chomika chińskiego CHO-9 do tworzenia kolonii, a stopień zahamowania zależał od rodzaju oraz stężenia badanego biopaliwa. Najsilniejsze działanie ograniczające zdolność komórek CHO-9 do proliferacji wykazywało biopaliwo II otrzymane z tłuszczu zwierzęcego oraz biopaliwo III otrzymane z posmażalniczego oleju roślinnego. Najsłabiej na zdolność tworzenia kolonii komórek CHO-9 działało biopaliwo IV otrzymane z surowego oleju rzepakowego. Wyniki sugerują, że biopaliwa mogą odgrywać rolę w mechanizmie molekularnym związanym z proliferacją komórek i odpowiedziami immunologicznymi, co powinno zostać potwierdzone w przyszłych badaniach genotoksyczności biopaliw.

Abstract

The four types of biofuels obtained by methanol transesterification of: rapeseed expired oil (BP I), waste animal fats (BP II), frying vegetable oils (BP III), and rapeseed oil (BP IV) were studied on the Chinese hamster ovary (CHO-9) cells using the clonogenic assay. Colony test results showed that tested biofuels inhibited the ability of CHO-9 cells to form colonies, depending on the type and concentration of the biofuel being tested. The strongest action in reducing CHO-9 cell proliferation capacity was demonstrated by biofuel II and biofuel III. Biofuel IV had the least effect on the ability of the CHO-9 cells to colonize. The results suggest that biofuels might play a role in molecular mechanisms associated with cellular proliferation and immune responses, that should be confirmed in the future studies on genotoxicity of biofuels.

Słowa kluczowe:
biopaliwa, test tworzenia kolonii, komórki jajnika chomika chińskiego (CHO-9), in vitro

Keywords:
biofuels, clonogenic assay, Chinese hamster ovary (CHO-9) cells, in vitro