

Cytotoxicity of Used Frying Oil Recovered by Different Adsorbents

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ABSTRACT

Adsorbents have been used as filtration aids for the effective recovery of repeatedly used oil, particularly in terms of physical characters such as color and odor. Efficacy in the reduction of the cytotoxicity of the used oil, however, remains to be confirmed. Palm oil was heated at 180 °C for chicken nugget deep-frying for 10 repeated frying cycles, with each cycle involving 4 hr of continuous batch frying with 8 min per batch. Three different filtration methods were applied at the end of each cycle and the recovered oils were examined for their cytotoxic effect on Mardin-Darbi canine kidney cell culture. The methods comprised filtration through filter paper without adsorbent aids (the control) and filtration with the aid of magnesol and bentonite adsorbent as treatment groups, respectively. The recovered oil samples from each group were classified into three levels according to the total polar material (TPM) contents (TPM < 20%, 20% ≤ TPM ≤ 25% and TPM > 25%). Cell proliferation was examined for cytotoxicity effects using apoptotic and clastogenic assays, with Hoechst 33342 and Giemsa staining, respectively. Based on the proliferation assay, the magnesol-treated group appeared to be less cytotoxic compared to the bentonite-treated and control groups with TPM contents of 20–25% ($P < 0.05$). No significant differences among treatment means were detected at TPM < 20%. The TPM contents of the magnesol-treated group, unlike those of the bentonite-treated and control groups, did not exceed 25% in the course of this study. Nuclear fragmentation as an apoptotic characteristic was observed while chromosomal aberration was not found. Active filtration with magnesol aids could be suggested as a potential refreshing means for extending the lifespan of used frying oil.

Keywords: used frying oil, adsorbent, bentonite, magnesol, cytotoxicity

INTRODUCTION

Under high heat and moisture, the physicochemical properties of frying oil are altered by oxidation, hydrolysis and polymerization resulting in the generation of toxic substances mostly classified as polar compounds and aromatic hydrocarbons (Picariello, *et al.*, 2009; Zhang, *et al.*, 2012). Polar materials, particularly reactive radicals, have been a major health hazard concern with repeatedly used frying oil; however, some

commonly identified compounds might not be factors determining the biological toxicity (Totani, *et al.*, 2008). Exposure to deteriorated frying oil has been found to be related to some crucial health problems, including metabolic alterations (Chao *et al.*, 2001; Chao *et al.*, 2007; Koch *et al.*, 2007; Chen *et al.*, 2008; Chiang *et al.*, 2011), atherosclerosis (Staprans *et al.*, 2005; Adam *et al.*, 2008; Xian *et al.*, 2012; Kummerow, 2013), hypertension (Soriguer *et al.*, 2003; Leong *et al.*, 2010; Jaarin *et al.*, 2011; Ng *et al.*, 2012), coronary heart disease

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(Kummerow, 2013) and cancer (Solovyan *et al.*, 1999; Lowe and Lin, 2000; Srivastava *et al.*, 2010; Chopra and Schrenk, 2011). Therefore, the total polar material (TPM) in frying oil has been applied as a regulatory index to maintain safety standards. However, the recommended standard level varies among countries and ranges from 24 to 27% in European countries (Stier, 2013). Such a recommended standard for the TPM content in frying oil in Thailand has not yet been seriously applied and thus should be pursued.

Although used frying oil potentially contains toxic substances, multiple reuses have been practiced to reduce costs, thereby giving rise to a potential consumer health hazard. Used frying oil has also been widely applied in animal feed with consequential toxic effects having been reported in poultry and swine (Engberg *et al.*, 1996; Ringseis *et al.*, 2007; Tavarez *et al.*, 2011; Zhang *et al.*, 2011). Such toxicity of the used oil could be indirectly transferred to consumers by consumption of the products derived from these livestock.

Filtration has been used to recover deteriorated, used frying oil thereby extending its lifespan. The efficacy of toxic substance removal has varied depending on the filter materials and conditions employed (Lin *et al.*, 2001; Bheemreddy *et al.*, 2002; Miyagi and Nakajima, 2003; Bhattacharya *et al.*, 2008; Farag and Basuny, 2009; Buczek and Chwialkowski, 2010; Sonkaew and Chaisena, 2012). Only particulates are removed by passive filtration using filter media such as filter paper, metal screens, plastic cloth and diatomaceous earth (Bheemreddy *et al.*, 2002) while active filtration with adsorbent aids can additionally remove unwanted substances, including color, odor, polar materials and aromatic hydrocarbons (Lin *et al.*, 2001; Bhattacharya *et al.*, 2008; Buczek and Chwialkowski, 2010). Different adsorbents have been commercialized following tests to remove specific chemicals; however, a biological toxicity test is still lacking to ensure the use of the recovered oil is safe. Therefore the aim

of this *in vitro* study was to evaluate the biological toxicity of repeatedly used frying oil samples that were recovered using different types of filter materials.

MATERIALS AND METHODS

Experimental design

The effects of oil filtration with three different filtration materials (bentonite adsorbent, magnesol adsorbent and filter paper without adsorbent) and TPM concentrations of $TPM < 20\%$, $20\% \leq TPM \leq 25\%$ and $TPM > 25\%$ on the cytotoxicity of repeatedly used frying oil were studied using a 3×3 factorial design with the filtration material and TPM as the main effects. Mardin-Darbi canine kidney (MDCK) cell culture was used for the *in vitro* cytotoxicity evaluation of the used oil samples. Apoptosis based on cell proliferation assay and nuclear staining and clastogenicity based on chromosome staining were examined.

Used frying oil sample

Three similar frying containers were installed for preparing used frying oil samples of three treatments using three different filtration procedures with either filter paper, bentonite (Sigma-Aldrich, Munich, Germany) or magnesol® (Dallas Group of America, Inc.; Jeffersonville, IN, USA), as filtration materials. Palm oil was used at 180 °C for chicken nugget deep-frying in an open frying system. Ten repetitive frying cycles were undertaken with each cycle consisting of multiple batches of 8 min of frying duration per batch to complete a total frying time per cycle of 4 hr. The nugget-to-frying oil ratio was 1:6 weight per weight. Multiple filtrations, each at the end of the frying cycle were applied using the different filtration materials for each different treatment group. Pre- and post-filtration collections of 4% volume per volume of frying oil samples were conducted. Each sample was split equally in half and stored in brown bottles, one kept at 7–10 °C

for TPM analysis and the other kept at -20 °C for later *in vitro* cytotoxicity testing. Following each sample collection, the frying oil was replenished with fresh oil.

Cell culture

MDCK, CRL-2936 (ATCC; Manassas, VA, USA) was cultured at 37 °C, 5% CO₂ atmosphere using Dulbecco's modified eagle medium (Life Technologies Corp.; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies Corp.; Carlsbad, CA, USA) and 1% penicillin-streptomycin as a complete medium. At confluence, subculture was performed at a 1:3 split ratio, using 0.25% trypsin/EDTA (ethylenediaminetetraacetic acid).

Proliferation assay

The cultured MDCK cells were trypsinized, washed and re-suspended in complete medium before being transferred into a 96-well tissue culture plate at 2.5×10^4 cells per well followed by incubation at 37 °C in 5% CO₂ atmosphere. After being incubated for 24 hr, the culture medium was changed to complete medium containing a 1% oil sample in 0.2% dimethyl sulfoxide (DMSO). A negative control was supplied with the same medium but without the oil sample. A blank was prepared by adding the complete medium containing 0.2% DMSO into an empty well without any MDCK cells. The culture plate was then incubated for another 24 hr.

Cell proliferation was measured by means of spectrophotometry using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc.; Kumamoto, Japan) using the supplier's recommended protocol. Absorbance at $\lambda = 450$ nm was measured by an ELISA plate reader (DTX880 multimode detector; Beckman Coulter Inc.; Brea, CA, USA). The proliferation index was calculated by dividing the sample optical density (OD) with the negative control OD. The results were reported as mean \pm SE.

Apoptotic assay

Apoptosis was evaluated based on nuclear morphology, including a fragmented and pyknotic nucleus. Preparation of the MDCK cell culture in a 96-well plate and the application of treatments were similarly conducted as previously mentioned in the proliferation assay. After 24 hr of incubation with the oil samples, the cells were rinsed with phosphate buffered saline (PBS), pH 7.4 before adding 200 μ L of 5 μ g.mL⁻¹ Hoechst 33342 in PBS, pH 7.4 in each well. The plate was light protected and incubated at room temperature for 20 min and then was observed under a phase contrast fluorescence inverted microscope (FSX100; Olympus Corp.; Tokyo, Japan).

Clastogenic assay

Giemsa staining was performed to examine the chromosomal aberrations, using a protocol modified from the report by Indart *et al.* (2007). Briefly, MDCK cells were cultured in 25 mL tissue culture flasks at 37 °C and in 5% CO₂ atmosphere, using the complete medium containing 1 μ g.mL⁻¹ phytohemagglutinin to promote cell division. After 24 hr, the medium was changed to the complete medium containing 1 μ g.mL⁻¹ phytohemagglutinin and 1% oil samples for 48 hr before adding 10 μ g.mL⁻¹ ethidium bromide. After 40 min of incubation, mitoses were arrested in the metaphase by the addition of 1 μ g.mL⁻¹ colchicine for 10 min, then washed, trypsinized and incubated in a hypotonic solution, 0.075 M KCl, for 20 min before fixation in 3:1 methanol:acetic acid. The cell suspension of 15 μ L was dropped on a microscope glass slide before heat fixation. Giemsa staining was then performed. The chromosomes were observed under a light microscope with oil immersion.

Statistical analysis

Differences among means were analyzed by two-way analysis of variance using the SPSS program (Version 21; SPSS Inc.; Chicago, IL, USA). Significant differences were tested at

the $P < 0.05$ level. A comparison of means was performed by Duncan's multiple range test.

RESULTS AND DISCUSSION

The cytotoxicity study, based on the WST-8 cell proliferation assay that was used in this study determines the activity of dehydrogenase that is directly proportional to the number of living cells (Heng *et al.*, 2010). A proliferation index of less than one means that the number of living cells receiving treatment was lower than in the untreated control owing to either growth retardation or increased cell death. As shown in Table 1, the overall proliferation index values were less than one suggesting that used frying oil samples were toxic to the cultured cells regardless of the applied treatments. The effects of TPM and the adsorbents on the proliferation index were respectively significant ($P < 0.05$) and highly significant ($P < 0.01$) with a very highly significant interaction between both main effects ($P < 0.001$) detected. The magnesol-treated groups was found to have a significantly higher proliferation index compared to the bentonite-treated and control groups at 20–25% TPM ($P < 0.05$). Significant differences were not detected at the level of TPM $< 20\%$. The recovered oil samples having TPM $< 20\%$ underwent only a few frying cycles and filtration treatments; therefore, the differences among treatments, if present, may not have reached the statistically significant level. The samples having 20–25% TPM underwent longer frying cycles and more filtration repeats. The significant differences in the cytotoxicity effect could therefore be the accumulative effect of the filtration treatments. Unlike the bentonite-treated and control groups, the magnesol-treated group was able to maintain the TPM level to not exceeding 25%.

Regardless of the adsorbent effect, the oil samples having a TPM level above 25% significantly reduced the proliferation index (0.47 ± 0.12) compared to the TPM levels of 20–25% (0.74 ± 0.32) and below 20% ($0.62 \pm$

0.28). However, differences were not significant between the latter two. This study suggests that approximately 25% TPM content is a critical level above which the hazard to the cells in the used frying oil is significantly enhanced. Such a level is in accordance with the recommended TPM standard whose threshold varies from 24% to 27% in several European countries (Stier, 2013).

Vegetable oil, used repeatedly at high temperature during deep-fry cooking is subjected to a series of chemical degradations yielding hundreds of volatile and non-volatile polar compounds (Gere, 1982; Zhang *et al.*, 2012). TPM has been accepted as the appropriate representative of these polar compounds. Parts of TPM generated by oxidation and peroxidation reactions under high heat with the presence of moisture and oxygen in open frying system are free fatty acids (FFA) and reactive radicals and studies in experimental animals demonstrated that reactive radicals generated from the oxidative degradation of used oil could cause depletions of body antioxidants such as α -tocopherol (Izaki *et al.*, 1984; Liu and Huang, 1995, 1996; Huang *et al.*, 2009) and ascorbic acid (Liu and Lee, 1998). The oxidized oil intake could therefore induce oxidative stress (Izaki *et al.*, 1984; Quiles *et al.*, 2002; Ringseis *et al.*, 2007; Zhang *et al.*, 2011) leading to tissue damage (Chiang *et al.*, 2011). TPM is therefore related to the health hazard of deteriorated oil and thus has been applied as a key standard safety index for cooking oil (Stier, 2013).

Such detrimental effects could well be anticipated in human beings as the human body was found capable of the uptake and incorporation of dietary oxidized lipids into serum chylomicrons (Naruszewicz *et al.*, 1987; Staprans *et al.*, 1994, 1996, 2003). Oxidized fat consumption has been found associated with the development of some vital diseases such as insulin resistance (Koch *et al.*, 2007), arteriosclerosis (Staprans *et al.*, 1996; Khan-Merchant *et al.*, 2002; Adam *et al.*, 2008; Xian *et al.*, 2012; Kummerow, 2013) and cancer (Ichinose *et al.*, 2004; Srivastava *et al.*, 2010;

Diggs *et al.*, 2011). Cell growth retardation and/or the promotion of cell death reported in the above findings in the proliferation assay reiterate the pathological effect of repeatedly heated frying oil in the development of such chronic diseases. Hence, food safety precautions should be applied for the use of used oil either as feedstuff for animals or as cooking oil for human food.

The cytotoxicity of repeatedly heated frying oil was further examined in this study by nuclear staining with Hoechst 33342. Pyknotic and fragmented nuclei, indicative of apoptotic cell death (Oberhammer *et al.*, 1993a, 1993b; Nagata, 2005) were expressed in the cells exposed to hydrogen peroxide (Figure 1) and used frying oil treatments (Figure 2b), which were scarcely observed in those of the negative control group (Figure 2a). The cytotoxicity of repeatedly heated frying oil was therefore involved with the induction of apoptosis. Apoptosis is a body defense mechanism for eliminating destructive, abnormal cells (Bursch *et al.*, 1992), which involves the up-regulation of several genes including those encoding various cysteine proteases and endonuclease (Orrenius *et al.*, 2011; Errami *et al.*, 2013). This results in DNA fragmentation (Oberhammer *et al.*, 1993b;

Zhivotovsky *et al.*, 1994; Errami *et al.*, 2013) accompanied by plasma membrane blebbing and cell shrinkage (Lane *et al.*, 2005; Coleman *et al.*, 2001), which are morphological characteristics of apoptotic cells.

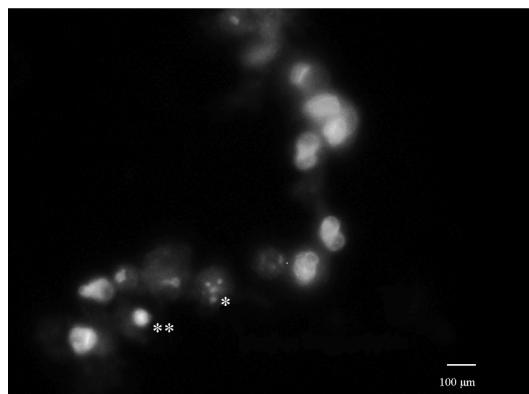


Figure 1 Apoptotic analysis using Hoechst 33342 staining on Mardin-Darbi canine kidney cells. Nuclear fragmentation (*) and a pyknotic nucleus (**), being key morphological features of the apoptotic cell, were visible (at 100 \times magnification) as a result of apoptotic induction by hydrogen peroxide exposure in the positive control group.

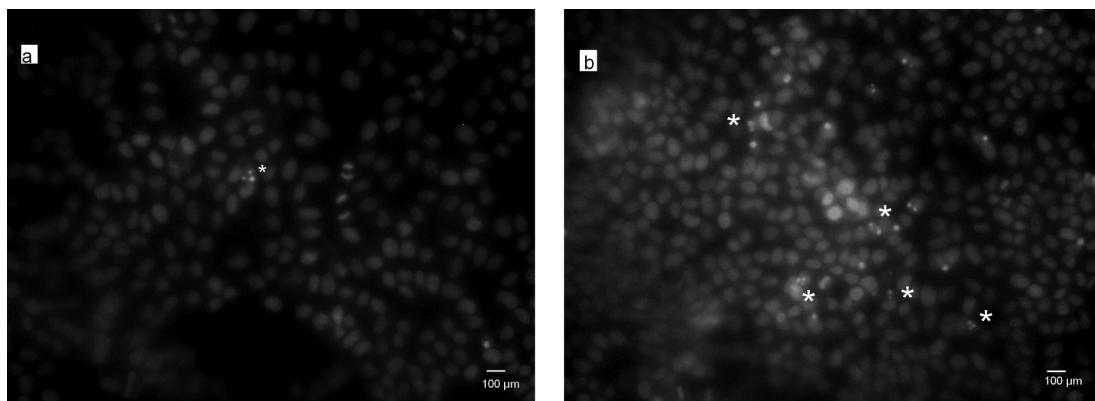


Figure 2 Fluorescence micrograph of Mardin-Darbi canine kidney cells stained with Hoechst 33342. Low fluorescent intensity with a few apoptotic cells were observed (at 63 \times magnification) in the negative control group without the administration of frying oil treatment (a) while intense fluorescence with a significant number of apoptotic cells was seen in the frying oil treatment groups (b). Nuclear fragmentation is indicated by *.

Accelerated apoptosis has been related to genotoxicity leading to possible mutation and cancer development (Solovyan *et al.*, 1999; Srivastava *et al.*, 2010; Chopra and Schrenk, 2011). Interruption of the cell cycle in S-phase has been reported suggesting a carcinogenic potential in mice (Pandey and Das, 2006). In line with the findings in the current study, exposure to repeatedly heated frying oil has been reported to cause apoptosis of several cell types including skin cells (Pandey and Das, 2006), pancreatic cells (Chiang *et al.*, 2011), hepatocytes (Pandey *et al.*, 2006), and immune cells (Fujimoto *et al.*, 2010; Chopra and Schrenk, 2011). However, the conventional metaphase analysis of Giemsa-stained chromosomes performed in this study did not reveal any chromosomal aberrations. A test with higher sensitivity such as multi-color chromosome painting (Ellard *et al.*, 1995, 1996) might be necessary for future investigation on the clastogenicity of repeatedly heated frying oil.

Although the repeated use of frying oil has been a crucial food safety concern, the cost-saving advantage from reuse is significant. Early disposal accounts for economic losses while extending reuse allows the accumulation of toxic, degraded compounds with an increased health hazard risk. Used oil deterioration in general has been identified based on its physical properties and the qualities of fried food. Foaming, viscosity, smoke point, odor and dark color are associated with a significant accumulation of degraded compounds (Paul and Mittal, 1996). Different filtrations have been employed to remove the compounds to correct these altered properties. Effective elimination of the dark color as well as the free fatty acids and viscosity by bentonite, magnesium silicate and pekmez earth has been reported (Maskan and Bagci, 2003). While regaining certain physicochemical properties has been achieved, the biological safety of the filtered used oil remains to be confirmed.

Considering the adsorbent effect reported in Table 1, a significantly higher proliferation

index ($P < 0.05$) was found in the magnesol group (0.77 ± 0.31) compared to the control (0.52 ± 0.29) and bentonite (0.51 ± 0.13) groups with no significant difference detected between the latter two. This could suggest that used oil samples treated with magnesol filtration were less cytotoxic than those of the other two groups. Active filtration with the aid of magnesol adsorbent might have superior capacity in the elimination of some toxic compounds generated during the repetitive frying cycles. Magnesol treatment was found to maintain the TPM content at a level not exceeding the 25% general safety standard throughout the 10 cycles, which accounted for a total of 40 hr of frying operation in this study. Within this experimental period, the used frying oil samples of the control and bentonite groups appeared to have TPM contents exceeding the 25% safety level. The greater efficacy of magnesol in containing the TPM level could be part of the explanation for less cytotoxicity based on the proliferation assay. Magnesol, a synthetic magnesium silicate, has been reported to effectively reduce FFAs (Bheemreddy *et al.*, 2002; Yilmaz and Erdim, 2012), which compose a significant part of the TPM content in repeatedly used cooking oil. In these other studies, consistent with the current study, magnesol was found to be superior to bentonite clay, (aluminum silicate) in reducing FFAs in chicken fat and blended chicken fat and vegetable oil. Reports on in-depth physicochemical analysis to compare these two adsorbents in the reduction of FFAs or the TPM content of repeatedly used frying oil have not been found.

The cytotoxicity of the refined used oil reported in the current study could provide additional support for further enforcing regulations regarding safe use and toxicity levels. However, the *in vitro* cytotoxicity testing conducted in the current study has some limitations, particularly regarding the short examination period permitted by the cell-based system. Therefore, to identify the chronic toxicity of the refined oil, *in vivo* long-term investigation might be used.

Table 1 Proliferation of Mardin-Darbi canine kidney cells in response to exposure to repeatedly heated frying oil recovered by different adsorbents containing different levels of total polar material (TPM) contents.

TPM	Adsorbent	Proliferation index ¹	P-value
Treatment effect			
< 20% (X)	Filter paper (C)	0.66±0.12	
	Bentonite (B)	0.62±0.05	
	Magnesol (M)	0.50±0.06	
20–25% (Y)	Filter paper (C)	0.37±0.02 ^a	
	Bentonite (B)	0.45±0.03 ^a	
	Magnesol (M)	0.98±0.06 ^b	
> 25% (Z)	Filter paper (C)	0.39±0.01	
	Bentonite (B)	0.49±0.04	
	Magnesol (M)	NA	
TPM effect			
< 20% (C+B+M)		0.62±0.28 ^c	
20–25% (C+B+M)		0.74±0.32 ^c	
> 25% (C+B)		0.47±0.12 ^d	
Adsorbent effect			
Filter paper (X+Y+Z)		0.52±0.29 ^e	
Bentonite (X+Y+Z)		0.51±0.13 ^e	
Magnesol (X+Y)		0.77±0.31 ^f	
Source of variation			
TPM			*
Adsorbent			**
TPM×Adsorbent			***

¹ Values shown as mean ± SE having different lowercase superscript letters within a column and section (treatment, TPM and absorbance) are significantly different ($P < 0.05$).

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; NA = Not applicable.

CONCLUSION

Magnesol appeared to be better than either bentonite adsorbent or filter paper at improving the quality of refreshed palm oil based on magnesol having the best TPM reduction capacity and the least toxicity to MDCK cells.

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