The genotoxicological evaluation of several local raw foods extracts on Chang liver cells by Single Cell Electrophoresis Assay

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Summary

Local raw foods such as the salted fishes, dried shrimps, anchovies and the shrimp pastes (belacan) have been used in many Malaysian cookings. In this study, the effects of those foods extracts on the DNA of the Chang liver cells were evaluated using the Single Cell Electrophoresis Assay (Comet Alkaline Assay). Percentage of damage to the DNA was calculated using manual scoring based on the severity of the DNA damage (tail moment). "Belacan" at 62.5 µg/mL showed the strongest damage to the DNA (100 ± 2.13%), followed by the salted fish (100 ± 8.6%), dried anchovies (21.67 ± 8.4%) and the dried shrimps (18.5 ± 3.4%). High salt content could be related to the genotoxicity. Further investigations should be carried out to determine their toxicological profiles to evaluate more of their potential hazards to health.

Keywords: local raw foods, genotoxicity, Chang liver cells, Comet Alkaline Assay

Introduction

Cancer can be regarded as a disease of cells; it is characterized by an excess of cells beyond the number needed for normal function of the body organ affected. Cancer almost always occurs in a specific body organ such as the lung, the liver or the breast and the causes of these different cancers are also different (AICR, 1997).

Cancer can be caused by various environmental factors that include electromagnetic radiation, chemicals, fibers, viruses and dietary components (AICR, 1997). There is sometimes a perception that the major cancer risk from diet comes from specific carcinogenic substances such as additives or contaminants in foods and drinks. However, based on the food consumption patterns, data on naturally occurring carcinogens and on pesticides residues and contaminants in foods, it has been estimated that a greater food-borne risk comes from carcinogens which are natural constituents of food (Scheuplein, 1990). Known or suspected dietary carcinogens contained in food before it is consumed, or produced endogenously during digestion, include heterocyclic amines (generated in meat cooked at high temperatures), N-nitroso compounds (arising from spoiled or fermented foods and proteins), polycyclic aromatic hydrocarbons (products of combustion in cooked food) and alcoholic beverages (AICR, 1997). Thus, regardless of how the risk assessment is done, most of the risks come from the food itself, not synthetic additives or contaminants.

Diet plays a major role in cancer etiology and prevention. In early 1980, Doll and Peto had correlated the relation between diet and cancer (Doll and Peto, 1981). Overall, there have been current available data to support direct relationship between cancer risk and intakes of total fat, alcohol, as well as obesity and certain preparation methods such as smoking, salting and pickling foods and high temperature cooking of meats (AICR 1997; Smith and Giovannucci, 1999; US Department of Health and Human Service, 1996; Ballard-Barbash, 1999; Longnecker...
Numerous types of genes are likely to be involved in human carcinogenesis such as genes that could influence the metabolic activation/detoxification, DNA repair, chromosome stability, activity of oncogenes or tumor suppressor genes, cell cycle control, signal transduction, hormonal pathways, vitamin metabolism pathways, immune function and receptor or neurotransmitter action (Sinha and Tseng, 1999; Martinez et al., 1999; Zhou and Blackburn, 1999; Combs and Clark, 1999).

Thus, to understand how nutrients and other diet-related factors can modulate the carcinogenic process through interactions with various genes is always essential. Dietary carcinogens such as aflatoxin B₁ and polycyclic aromatic hydrocarbons (PAHs) can alter DNA by forming adduct (Strickland and Groopman, 1995). Meanwhile, the consumption of carotenoid-rich carrot and tomato juices can reduce oxidative DNA damage in human lymphocytes by various mechanisms (Pool-Zobel et al., 1998). These days, many food components were reported to be potentially genotoxic (Manson and Benford, 1999). Genotoxicity refers to an adverse effect on the genetic material (DNA) of living cells. Several various techniques for detecting DNA damage, as opposed to the biological effects (e.g. micronuclei, mutations, chromosomal aberrations) that results from DNA damage have been used to identify substances with genotoxic activity. However, the Comet Assay or Single Cell Electrophoresis Assay that was introduced by Ostling and Johanson (1984) and modified by Singh et al. (1988) has been widely used in the genotoxicological study. Comet assay often used to identify agents with genotoxic activity. The term “comet” is used to identify the individual cell migration patterns produced by this assay. Relative to other genotoxicity tests, the advantages of the assay includes its sensitivity for detecting low levels of DNA damage, the requirement for small number of cells, its flexibility, its ease of application and the short time to complete a study (Tice et al., 2000).

In this study, Human Chang liver cells were used to evaluate the toxicity effect of those local food products. Human Chang liver cells were chosen to be used in this study because of its characteristics where it is rich with enzymes such as cytochrome P450 which are responsible in the biphasic metabolism of the xenobiotic compounds that enter the body (Boobis et al., 1981).

Findings reported that there were genotoxicity effects and maximum chromatid damages from salted fish and meat in India (Taj and Nagarajan, 1994). Previous study had also shown that salted fishes are one of the risk factors of lung cancers in Guandong-China besides consumptions of alcohol, family history and smoking (Wang et al., 1996). Based on these preliminary screening, Yuan et al. (2000) had suggested that salted fishes can increase the risk of nasopharyngeal cancer. Moreover, based on another epidemiological research in Malaysia, consumptions of salted fishes did show positive associations with nasopharyngeal cancer patients (Armstrong et al., 1998).

Hence, this study was conducted to evaluate the genotoxic potential of those local raw processed food products (salted fishes, dried shrimps, anchovies and the shrimp pastes) using the Alkaline Comet Assay on Chang liver cell.

Materials and Methods

1. Food samples

All the food samples (anchovies, salted fishes, shrimp pastes and dried shrimps) were purchased from Chow Kit Wet Market, Kuala Lumpur on the same day. The samples were cut to small pieces and ground into powdered materials.

2. Extraction of food samples

The ground powder of the food samples (200 g) was soaked successively in 500 mL of methanol (Chemical Industries, Malaysia) for two days. Each of the mixtures was then filtered and evaporated using a rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 50°C under reduced pressure to evaporate the solvent from the extracts. The resulting pellet was the subjected under a vacuum in a freeze-drier (Heto LyoLab 3000, Denmark) and the dry powdery extract was kept at 4°C in an air-tight jar prior to bioassay activity.

3. Reagent and cells

Human Chang liver cells were obtained from ATCC (Rockville, MD USA) and cultured as described (ATCC Catalogue Details No CCL 13, 2003). Cells were grown as monolayer in T-25 cm² culture flask. Media were supplemented with 2.0 g/L sodium bicarbonate, antibiotics; 100 units of penicillin/mL, 100 µg of streptomycin/mL and 10% fetal bovine serum (FBS). Cell culture media and their supplements were purchased from Life Technologies, Gibco BRL Products (Rockville, MD USA). The cell cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C and were harvested when they reached 80% confluence to maintain exponential growth. For bioassay activity, methanol extracts of each sample were dissolved in 5% dimethyl sulfoxide (DMSO) (Chemical Industries, Malaysia) and media RPMI-1640 (Flowlab UK) to a final concentration of 10 mg/mL. These solutions were then filtered using sterile syringe filter 0.45 µm. Hydrogen peroxide at 100 µM was used as the positive control for MTT assay and 0.1 nM for alkaline comet assay.
4. The MTT [3-(4,5-diemethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazoli-um] cytotoxicity assay (Mosmann, 1983)

Human Chang liver cells were used to determine the cytotoxicity effect of each of the food sample. The cell monolayer in exponential growth was harvested using 0.025 \( \text{g/mL} \) trypsin (0.025 \( \text{g/mL} \) trypsin, 1mM EDTA 4Na) and single cell suspension was obtained by repeated pipetting. The cells were counted with a hemacytometer and 100 \( \mu \text{L} \) of single cell suspension at 5 \( \times \) 10^4 cells per mL was loaded into the 96-well plates (Nunclon\textsuperscript{TM}, VWR International Inc., MD USA) and incubated for 24 h at 37\( ^\circ \text{C} \) in a humidi- fied atmosphere of 5\% CO\(_2\). For treatment, media were discarded and 200 \( \mu \text{L} \) of the test extract for each sample was loaded into the 96-well plates ranging from high to low concentrations. Treatment at 500 \( \mu \text{g/mL} \) and 4 \( \text{mg/mL} \) were used to determine their cytotoxic effect. After 72 h incubation, 20 \( \mu \text{L} \) of the MTT (Sigma Chemical Co., St. Louis, MO USA) solution was added to each well of 96 well plates and incubated for 4 h at 37\( ^\circ \text{C} \) in a humidi- fied atmosphere of 5\% CO\(_2\). At the end of the incubation period, the media was discarded and 100 \( \mu \text{L} \) of DMSO was added to solubilize the formazan crystal. The plates were incubated for 15 min to dissolve the entire crystal.

Fig. 1 Percentage of the viability Chang cells when treated with Malaysian food extracts at different concentrations (a = 500 \( \mu \text{g/mL} \) and b = 4 \( \text{mg/mL} \)) *significant compared to negative control at \( p<0.05, n=3 \).

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**Graph a**

**Graph b**

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formed. The absorbance was measured at 570 nm with a microplate reader. The metabolic activity of the cells was measured after 72 h of treatment where the MTT salt was reduced to a coloured formazan by the active viable cells with dehydrogenase enzyme.

5. The Comet Assay

Human Chang liver cells were used in this study and seeded at $4 \times 10^5$ cells per mL (2 mL each well) into 6-well plates (Nunclon™, VWR International Inc. MD) and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂. Media was discarded and test extracts at concentration of 62.5 µg/mL (IC₁₀) were added for 24 h treatment. Negative control contained the media itself without any extract. After 24 h treatment, cells were washed using phosphate buffer saline (PBS) and trypsinized to detach the cells. Each well was done in triplicates and transferred to eppendorf tubes. Cells were centrifuged at 2,500 rpm for 5 min twice and the second time is with PBS to wash the cells. Frosted slides were prepared with a layer of normal melt agarose. After the agarose gel had solidified, the slides are placed generally for at least 1 h in a lysis solution consisting of high salts and detergent (100 mM ethylenediaminetetraacetic acid (EDTA), 2.5 M sodium chloride, 10 mM Trizma base, adjusted to pH 10, with 1% Triton X-100 was added just prior to use. Then, slides were incubated in alkaline (pH > 13) electrophoresis buffer for 20 minutes to produce single stranded DNA. The alkaline solution consisted of 1 mM EDTA and 300 mM sodium hydroxide. After alkali unwinding, the single stranded DNA in the gel was electrophoresed under alkaline conditions at 25 V and 300 mA for 20 min to produce comets. The alkaline buffer used during electrophoresis was the same pH > 13 buffer used during alkali unwinding. Next, the alkali in the gels was then neutralized by rinsing the slides with a suitable buffer (Trizma at pH 7.5) three times for 5 minutes each. Lastly, slides were stained using fluorescent dye (ethidium bromide). Slides were analyzed using Leitz Laborlux Epifluorescence Microscope, Germany equipped with 515 barrier filter and 560 emission filter.

Results and Discussion

Because DNA damage is associated with cell death, it is critical that the highest dose tested does not induce excessive cytotoxicity and that cytotoxicity be evaluated concurrently with each comet experiment. In this study, preliminary cytotoxicity measurement with the tetrazolium reduction assay (MTT) was performed. It is recommended in the Comet Assay Guidelines to avoid the testing of doses that decrease the viability by more than 30% (Anderson et al., 1998; Henderson et al., 1998; Tice et al., 2000). In order to fulfill these requirements and to be able to discern between cytotoxicity and genotoxicity, a short incubation time of 24 h at 37°C had been applied.

Based on the graph of the cells viabilities, at 500 µg/mL (Fig. 1a), all the extracts showed 20% of reduction in the cell viabilities. The percentage of reduction remained clear when higher concentration of the extract was used (4 mg/mL). Thus, in this study, treatment for comet assay to evaluate the genotoxic effect was chosen at 62.5 µg/mL. Moreover, the acceptable daily intake for these local foods products were reported to be less than 5 g/mL (Ministry of Health 2005, unpublished report).

The genotoxicity effects of all the food samples were determined using a standard score (Singh et al., 1988) to distinguish the different levels of the damage of the cells. Score was given based on the tail moment of the comet. Score 0 was given to the cell without the DNA damage while score 1, 2, 3 and 4 were given according to the severity of the DNA damage. Based on the graph, at 62.5 µg/mL, the methanol extracts of dried anchovies and dried shrimps showed the least of percentage of cell damage; however, at the same concentration, the methanol extract of anchovies were at the score 1 (17.68 ± 6.86%), and score 2 (2.67 ± 4.62%) whereas for dried shrimp, there was only damage at score 1 with 18.5 ± 6.86%.

However, at the same concentration, the methanol extracts of salted fish and shrimp pastes caused total damage of the cells. Damaged cells that were caused by salted fish were at score 2 (5.00 ± 4.58%), score 3 (86.33 ± 5.77%) and score 4 (12.00 ± 4.58%). Meanwhile, the damaged cells for the methanol extracts of the shrimp paste were at score 3 (84.67 ± 2.52%) and score 4 (14.33 ± 1.15%). These extracts caused severe damages for the
majority of the cells.

Dried shrimp and anchovies showed some protective effect from the DNA damage. This could be due to the presence of high amount of carotenoid and retinol in the dried shrimps and also the calcium in anchovies that could have some antioxidant potential. Carotenoids are efficient antioxidants in scavenging singlet molecular oxygen and peroxyl radicals (Sies and Stahl, 1995).

However, the damage of the cells could be due to the high amount of salt. High salt content can also cause the death of the cells in mechanisms such as osmosis or alteration to the homeostasis of the cells. A bacterium named *H. pylori* was found in the stomach and can increase the risk of gastric cancer because of the damaging effect on the gastric mucosa, leading to an increase in regenerative cell duplication (Weisburger, 2000). Similarly, salt has the same effect in the stomach as the bacteria (Tsugane et al., 1994; Fox et al., 1999). Findings showed that high concentrations of salt might also increase the incidence of gastric cancer in laboratory animals. The mitogenic response would also favour the progression of the cells towards neoplasia (Cohen and Roe, 1997). In addition, high salt concentrations are also capable of inducing chromosomal damage through indirect genotoxic action. It is most likely that the chromosomal changes observed from the salted foods are due to the clastogenic compounds present (Cohen and Roe, 1997).

### Conclusion

This study shows that anchovies and dried shrimp are not genotoxic agents towards Chang liver cells whereas salted fish and shrimp pastes (belacan) showed total damages to the DNA. This indicated that those food items might be potential genotoxic agents. Further research should be carried out to determine more of their toxicological profiles.

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