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FULL PAPER

# Mutagenicity of modelled-heat-treated meat extracts: Mutagenicity assay, analysis and mechanism of mutagenesis

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## Abstract

**Cooking of meat usually aims in producing microbiologically safe food suitable for human consumption. However, doing so at such high temperatures may produce some cooking toxicants or mutagens. The objectives of this study were to investigate the mutagenicity of modelled-heat-treated meat after different cooking methods (boiling, pan-frying and charcoal grilling) using Ames Salmonella typhimurium mutagenicity assay. In addition, the content of benzo[a]pyrene (B[a]P) in the meat extracts prepared under different cooking methods were measured using HPLC. In a trial to investigate the causes behind the mutagenicity of different meat extracts, HepG2 cell line was exposed to different modelled-heat-treated meat extracts. mRNA expression levels of various phase I and II xenobiotic metabolizing enzymes (XMEs) were examined using real time PCR. The results obtained declared that pan-fried and charcoal grilled-meat extracts significantly induced production of histidine+ revertants in the Ames mutagenicity assay. Grilled-meat extracts had the highest residual concentrations of B[a]P followed by pan-fried-meat, boiled meat and raw meat extracts, respectively. Induction of XMEs especially CYP1A1, CYP1A2 and NQO1 may contribute to the mutagenic ability of these extracts. It is highly advisable to control cooking temperature, time and method in order to reduce cooked-meat mutagens.**

Key Words: Heated meat, mutagenicity, B[a]P, xenobiotic metabolizing enzymes

## Introduction

The aim of cooking is to produce bacteriologically safe food with optimal sensory properties and the minimum content of possibly harmful substances. However, cooking and food processing at high

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temperatures have been shown to generate various kinds of cooking toxicants. Today there is a growing concern about the impact of these substances on human health. Human exposure varies among individuals due to dietary habits, meat cuts and cooking practice. Such toxicants include polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines and nitrosamines, which are produced due to heat treatment of meat[25].

PAHs are a group of more than hundred aromatic compounds that are formed due to the incomplete combustion of organic materials. Several individual PAHs like benzo[a]pyrene (B[a]P), are associated with carcinogenic, mutagenic and genotoxic effects in animal experiments. B[a]P is considered a group 1 carcinogenic compounds to human[11].

Cytochrome P450 (CYP) 1A1 and 1A2 are major xenobiotic metabolizing enzymes (XMEs) responsible for the metabolism of procarcinogenic and environmental pollutants such as PAHs[5]. The resultant metabolites undergo conjugation, elimination, and detoxification reactions via phase II metabolizing enzymes, including UDP-glucuronosyltransferases (UGT) and NAD(P): quinone oxidoreductase 1 (NQO1). The balance between xenobiotic bioactivation and elimination is key in controlling the incidence of mutagenesis and carcinogenesis of certain chemicals like B[a]P[6].

Several epidemiological studies had shown close association between the incidence of gastrointestinal tract (GIT) cancers and dietary ingestion of PAHs. For instance, studies done in China and Iran were in agreement with this hypothesis[28,13]. Additionally, Sinha *et al.*[25] showed that consumption of well-done red meat was associated with increased risks for adenoma of the sigmoid and descending colon. On the other hand, findings from Tabatabaei *et al.*[27] and Cross *et al.*[4] did not support this close association between B[a]P exposure through red meat intake and colorectal cancers.

Due to this debate on this important issue associated with public health, we investigated the mutagenic activity of different modelled-heat-treated meat extracts (boiled, pan-fried and charcoal grilled-meat) using Ames *Salmonella typhimurium* mutagenicity assay. Furthermore, we analyzed the aforementioned extracts for their contents from the pro-mutagen, and pro-carcinogen B[a]P. Liver is the target organ of xenobiotic metabolism and detoxification and HepG2 cells and their derivatives are commonly used as a model system for studies of liver metabolism and toxicity of xenobiotics, the detection of environmental and dietary cytotoxic and genotoxic agents. HepG2 cells are able to activate and detoxify xenobiotics and therefore reflect the metabolism of xenobiotics in the human body better than other metabolically incompetent cells used in conventional *in vitro* assays[19]. Thus, in order to explain the mutagenicity of the heated-meat extracts, human liver HepG2 cells were exposed to these extracts, and the mRNA expression of the concerned phase I and II XMEs were examined using real time PCR.

## Materials and Methods

All experiments were performed according to the guidelines of Hokkaido University Institutional Animal Care and Use Committee.

*Chemicals and reagents:* All of the test reagents used were of HPLC grade, including those described below. Benzo[a]pyrene, TRI reagent and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Oligo (dT) primer, ReverTra Ace and reverse transcriptase (RT)-buffer were purchased from TOYOBO (Osaka, Japan). Primer sets were purchased from Invitrogen (Carlsbad, CA, USA). NADPH, glucose-6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), and co-factor S9 were from Oriental Yeast (Tokyo, Japan). All other reagents were of analytical grade or the highest quality available and purchased from Wako Pure Chemical

Industries (Tokyo, Japan).

*Preparation of meat samples:* Lean beef cut (15% fat) was purchased from local market in Sapporo, Japan. The beef cut was divided into 20 patties samples (50 g, 0.5 × 4 inches each). The obtained samples were grouped into four groups (5 samples) each. The first group was remained unprocessed as control, called later as raw-meat. The second group was boiled at hot water (water temperature is 100°C) for 20 min and named hereafter as boiled meat. The third group was pan-fried in vegetable oil at 180°C, internal temperature 80°C for 8 min on each side, (total time is 18 min), and named hereafter as pan-fried meat. The fourth group is grilled under open-flame charcoal and named hereafter as grilled meat. The distance between the fire and the meat samples was 15 cm. The internal temperature of the meat cut was 90°C for 6 min on each side, (total grilling time was 12 min). All samples were normal done meat. The meat samples were kept frozen at 0°C in dark till analysis.

*Preparation of meat extracts:* Meat extraction was done by the method described before[12] with slight modifications. Briefly, about 10 g of each meat sample was extracted with approximately 25 mL of 1 M KOH ethanol solution, and saponified for 10 h at 60°C. The saponified solutions were then shake-extracted three times with n-hexane and the resulting hexane fractions were run through granular sodium sulfate, evaporated using a rotary evaporator and purified using silica gel column chromatography (2 g of 5% water containing silica gel). The fractions obtained were eluted by 100 mL of acetone/hexane = 1/99 (v/v), dried under a gentle nitrogen stream, and re-dissolved into either 500 μL of di-methyl sulfoxide (DMSO) for mutagenicity and cell culture experiments or in 500 μL of methanol for B[a]P content using HPLC.

*Ames mutagenicity assay:* Salmonella mutagenicity assay was performed according to the method described by Ames *et al.*[2] with slight modifications. Briefly, *Salmonalla typhimurium* strain TA98, which is sensitive to frame shift mutations, was used. One milliliters of the reaction mixture containing 10 mg of non-treated rat S9 was prepared according to our previous modified method[5], 10 mM G-6-P, 100 μL of different modelled-heated-meat extracts, or B[a]P (10 nM) in DMSO as positive control[8], 1 mg co-factor S9 mixture. The reaction was started by adding 20 μL of a mixture of 50 mM NADPH and 200 U/mL of G-6-PDH. After incubation for 20 min at 37°C, the reaction was terminated by addition of top agar (maintained at 45°C). The tubes were then immediately plated onto minimum glucose plates (2% glucose, 15% agar) in duplicate and incubated at 37°C for 48 h. Histidine-independent revertants were scored manually using a colony counter. Background colony formation was consistently between 20 and 30 colonies/plate.

*Quantification of B[a]P in heat-treated meat extracts:* B[a]P was analyzed using HPLC[7]. HPLC analyses were performed using a Shimadzu LC20 series (Kyoto, Japan) equipped with a fluorescence detector (RF-10AxL) and a ZORBAX Eclipse PAH (2.1 × 150 mm, 3.5lm, Agilent). Quantification of B[a]P was determined from calibration curves made by standards (Accus Standard Inc.).

*Cell line and culture condition:* Human hepatoma cell line HepG2, obtained from RIKEN Cell Bank (Tsukuba, Japan), was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified incubator with 5% $CO_2$. Cells were seeded in 60 mm collagen-coated dishes, sub-cultured twice a week, and subsequently grown to 80–90% confluence. Cells were exposed to different heated-meat extracts dissolved in DMSO (1 ng/g) or 10 nM B[a]P (this concentration is physiologically relevant to human consumption[3,8]) in serum-free medium for 24 h. After cell treatments for 24 h,

**Table 1. Primer sequences of the target genes used in this study**

| Target | Sequence |
|---|---|
| Human CYP1A1 | F 5′-CTATCTGGGCTGTGGGCAA-3′<br>R 5′-CTGGCTCAAGCACAACTTGG-3′ |
| Human CYP1A2 | F 5′- CATCCC CCACAGCACAACAA-3′<br>R 5′- TCCCACTTGGCCAGGACTTC-3′ |
| Human CYP2A6 | F 5′-ATGGCCTCCCTGTACCACATC-3′<br>R 5′-TGTTGCGCTCAATCTCCTCCT-3′ |
| Human UGT1A6 | F 5′-CATGATTGTTATTGGCCTGTAC-3′<br>R 5′-TCTGTGAAAAGAGCATCAAACT-3′ |
| Human NQO1 | F 5′- GGATTGGACCGAGCTGGAA-3′<br>R 5′- AATTGCAGTGAAGATGAAGGCAAC-3′ |
| Human β-actin | F 5′- CTGGCACCCAGGACAATG-3′<br>R 5′-GCCGATCCACACGGAGTA-3′. |

the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS).

*Cell viability assay:* The cell viability was assayed using the CCK-8 assay (Sigma-Aldrich) by measuring the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye.

*RNA extraction and cDNA synthesis:* Total RNA was extracted using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (DYMO, Stamford, USA). The RNA quality was estimated by the 260/280 nm and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis.

The cDNA was synthesized according to our previous report[7]. In brief, a mixture of 5.0 μg of total RNA and 0.5 ng of oligo dT primer in a total volume of 24 μL of sterilized ultra-pure water was incubated at 70°C for 10 min and then removed from the thermal cycler (TaKaRa, Japan). The volume was increased to 40 μL with a mixture of 4 μL (5X) RT-buffer (Toyobo Co., Ltd, Osaka, Japan), 8 μL 10 mM dNTP, 2 μL water, and 2 μL reverse transcriptase (Toyobo Co., Ltd). The mixture was then re-incubated in the thermal cycler at 42°C for 45 min and at 90°C for 10 min to prepare the cDNA.

*Quantitative real-time polymerase chain reaction:* Quantitative real-time PCR for the mRNA levels of human phase I and II XMEs was performed using StepOne™ Real-Time PCR System (Applied Biosystems). The primer sequences were described in table 1[21,1]. PCR reactions were set in 10 μL volumes according to the protocol described in our previous report[6]. Briefly, the PCR reaction mixture was prepared with SYBR® qPCR Mix (TOYOBO), 10 μM of each primer, 600 ng cDNA and 50x ROX reference dye and RNase-free water. The mixture was made up to a final volume of 10 μL. The reaction cycle comprised an initial holding stage at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60 °C for 1 min and extension at 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis confirmed the amplification of a single amplicon of the expected size as well as the absence of primer dimers and genomic DNA amplification. β. actin was used for normalization in the comparative Ct method. β. actin expression is stable in HepG2 cells, The Ct value among control and treated cells ranged between 16.17 to 16.32. Furthermore, β. actin is frequently used as a housekeeping gene in real-time PCR in other

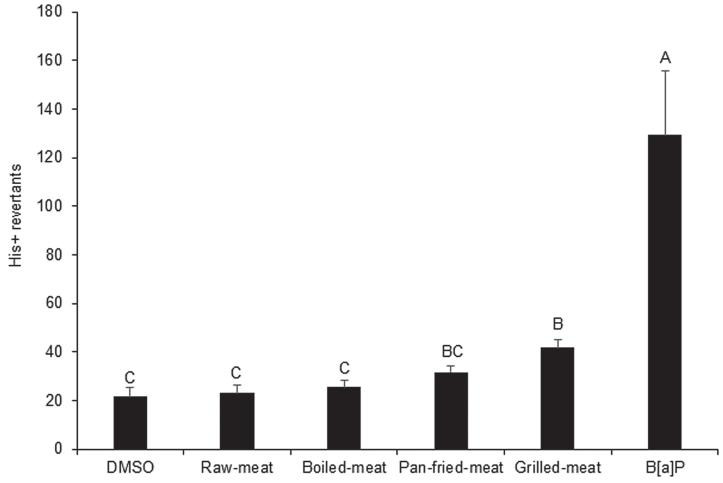


**Fig. 1. Mutagenicity of different modelled-heat-treated meat extracts and B[a]P.** The number of histidine+ revertant colonies reflects the mutagenic ability of different modelled-heat-treated meat extracts and B[a]P in *Salmonella typhimurium* TA98 mutagenicity assay. The data represent the mean ± SD (n = 5) for each modelled heat-treated meat as well as raw meat. Identical letters are not significantly different from each other ($P < 0.05$).

reports[21,1].

*Statistical analysis:* Statistical significance was evaluated using Tukey–Kramer HSD test and Spearman's test using (JMP statistical package, SAS Institute Inc., Cary, NC, USA). A P-value $<$ 0.05 was considered to be significant.

## Results

In this study, we tested the mutagenicity of the modelled-heat-treated meat extracts in the Ames mutagenicity assay. The obtained results showed that grilled-meat extracts had the highest ability to produce histidine revertants followed by pan-fried meat extracts as clear in the figure 1. Both raw and boiled-meat extracts did not have any significant difference with the control one (Fig. 1).

In order to explain the high mutagenicity recorded in some of the tested heat-treated meat extracts, B[a]P residual concentrations were measured using HPLC. The recorded results showed that the mean ± SD values were 0.04 ± 0.02, 0.14 ± 0.05, 0.98 ± 0.12 and 2.655 ± 0.38 ng/g in raw, boiled, pan-fired and charcoal grilled-meat extracts, respectively (Fig. 2A). Scatter plot between B[a]P content and mutagenic activation ability in the examined meat extracts showed a positive correlation ($R^2 = 0.868$) ($P < 0.0001$) (Fig. 2B).

In order to explain the possible mechanism behind the mutagenicity of the heat-treated meat extracts, human HepG2 liver cell lines were exposed to these extracts and mRNA expressions of phase I and II XMEs were investigated using real-time PCR. At first, there were no alteration in HepG2 cell viability after exposure to heat-treated meat extracts (data not shown). The modulatory effects of heat-treated meat extracts on the mRNA expression level of various phase I and II xenobiotic metabolizing enzymes were recorded in figures 3 and 4. Interestingly, we observed that grilled-meat and pan-fired meat extracts induced phase I enzymes especially, CYP1A1 and CYP1A2 (Figs. 3A, B, C) and phase II enzymes especially, NQO1 (Fig. 4A, B) mRNA expression. The level of induction was significantly higher ($P < 0.05$) than raw and boiled meat extracts, but lower than that of B[a]P (Figs. 3 & 4).

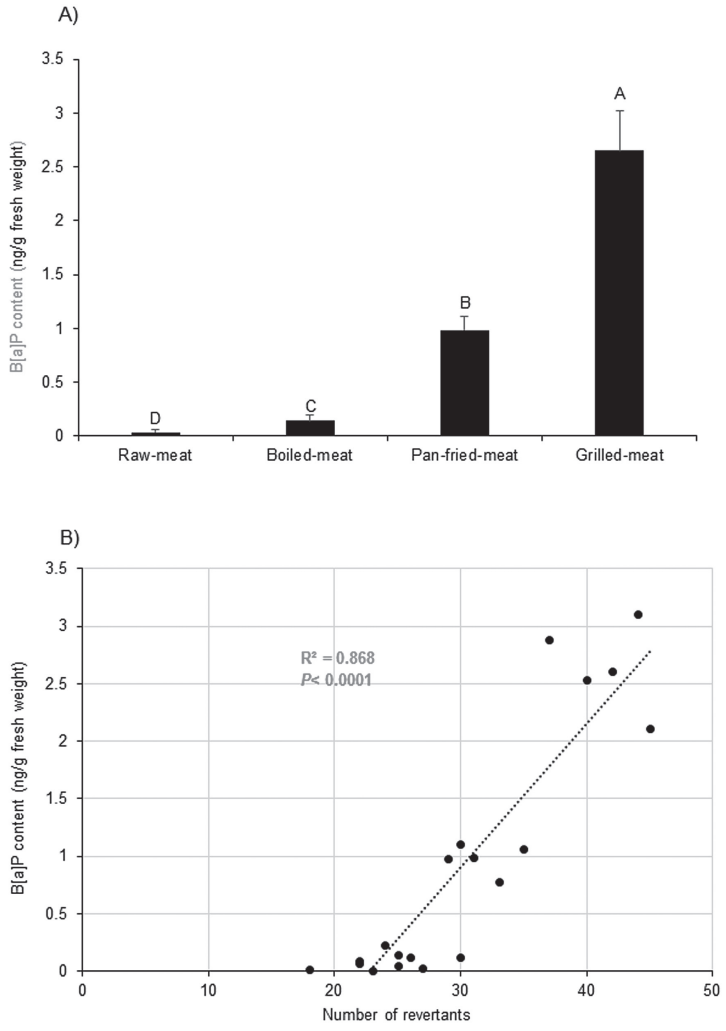


**Fig. 2. Relationship between B[a]P content and mutagenicity of different modelled-heat-treated meat extracts.** A) B[a]P content in the different modelled-heat-treated meat extracts. B[a]P content (ng/g fresh weight) was measured using HPLC. The data represent the mean ± SD (n = 5) for each modelled-heat-treated meat as well as raw meat. Identical letters were not significantly different from each other ($P < 0.05$). B) Scatter plots between B[a]P content (ng/g) and mutagenic activation ability of each modelled heat-treated meat extracts (His+ revertants) (Spearman's test $P < 0.0001$).

## Discussion

Meat is the most important source of animal-derived protein. It supplies the consumers with the essential amino acids and B-complex vitamins. At the same time, meat is one of the dietary sources, which contribute to one-third of the global variation of human cancer rates[26]. This study investigated the mutagenicity of heated-meat extracts prepared by different methods. The obtained results declared that grilled-meat extracts induced significant production ($P < 0.05$) of histidine revertants in the Ames-mutagenicity assay, twice higher than the negative control, and 1/3 like the revertants produced by B[a]P strong mutagen and carcinogen (Positive control) followed by pan-fried meat extracts (although not significant) as clear in the figure 1. In line with our finding, several epidemiological studies suggested that frequent consumption of well-done heated meat, especially the intake of PAHs, particularly, B[a]P, naturally generated in muscle meat during cooking, was strongly associated with human carcinogenesis at multiple sites,

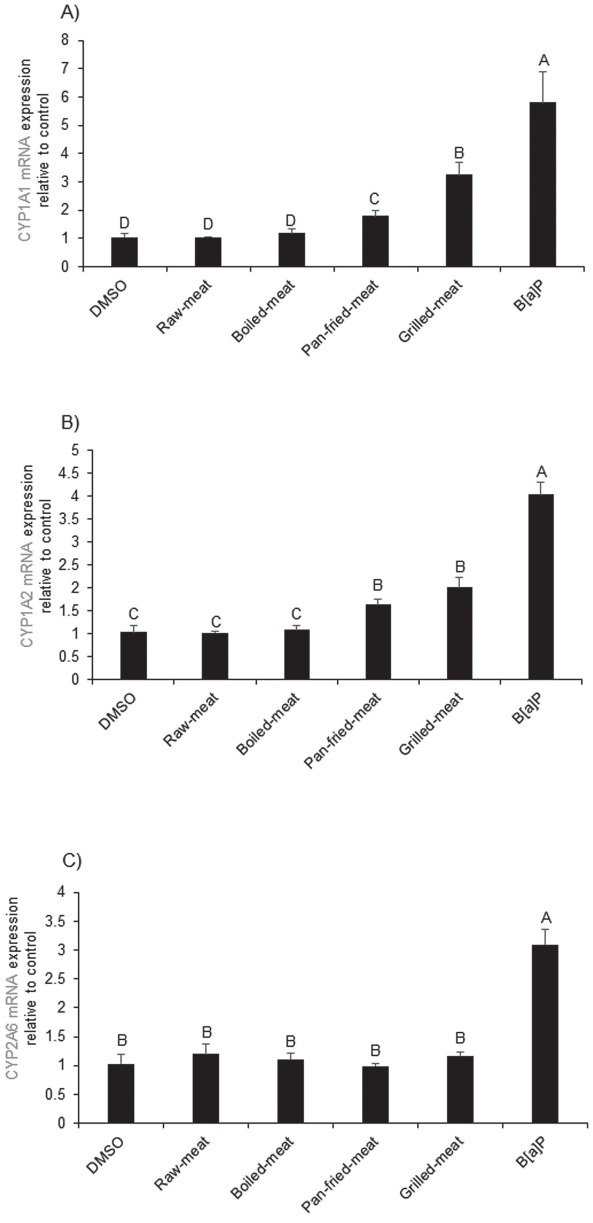


**Fig. 3. Expression of phase I enzyme mRNA in HepG2 human cells exposed to different heat-treated meat extracts.** The effect of different heat-treated meat extracts on A) CYP1A1, B) CYP1A2, C) CYP2A6 mRNA expression in HepG2 human cells as determined by real-time RT-PCR. Data are presented as the mean ± SD. Identical letters represent expression levels that are not significantly different from each other ($P < 0.05$).

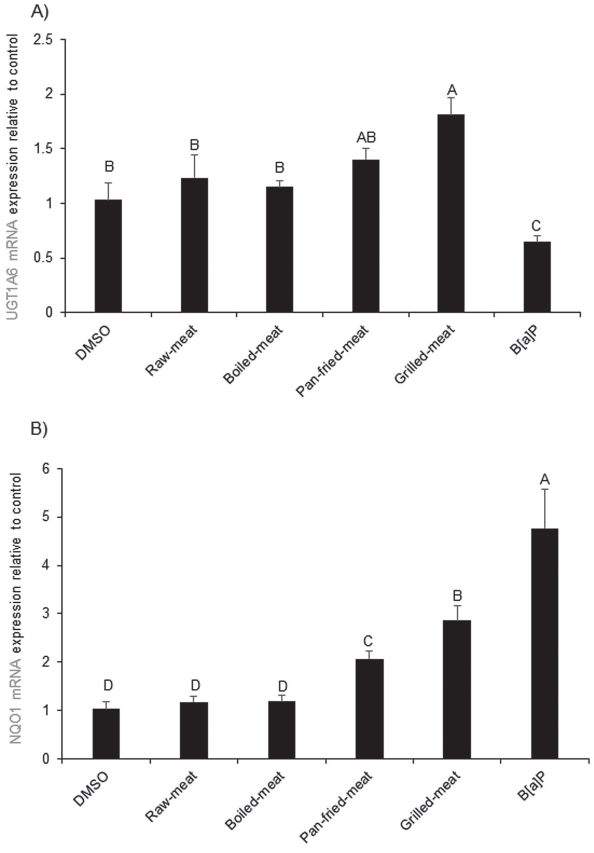


**Fig. 4. Expression of phase II enzyme mRNA in HepG2 human cells exposed to different heat-treated meat extracts.** The effect of different heat-treated meat extracts on A) UGT1A6, B) NQO1 mRNA expression in HepG2 human cells as determined by real-time RT-PCR. Data are presented as the mean ± SD. Identical letters represent expression levels that are not significantly different from each other ($P < 0.05$).

such as breast, colon, rectum, and prostate[16]. In addition to the type of meat, the processing methods and cooking conditions greatly affect the generation of PAHs, especially, B[a]P[15]. By analysis of various modelled-meat-extract samples for their B[a]P contents, the obtained results recorded similar trend to the mutagenic activation ability. Charcoal grilled-meat extracts had the highest B[a]P residual concentrations followed by pan-fried, boiled and raw meat extracts, respectively. Several researchers had studied the effect of cooking method and temperature on the release of PAHs, particularly B[a]P. Our results were in agreement with Chung *et al.*[3], who observed that charcoal-barbecued chicken meat had B[a]P concentrations higher than roasted and raw chicken meats, the range of B[a]P concentration in the charcoal cooked meat was 0.09–6.94 ng/g. In addition, Reinik *et al.*[22] estimated the concentrations of

B[a]P and 11 other PAHs in 322 commercial, meat products and 14 home-grilled meat samples and found that highest PAH concentrations were detected in home-grilled pork samples. Kazerouni *et al.*[14] obtained similar results when they analyzed meat samples cooked by different techniques for PAHs. The highest levels of B[a]P were found in grilled and barbecued steaks and chicken. Moreover, Larsson *et al.*[18] studied the levels of 22 PAHs in grilled meat and meat products and their results revealed that PAH levels are strongly dependent on the method of cooking and type of heat source used. The grilling of sausage in a direct flame resulted in extremely high PAH levels up to 212 ng/g B[a]P.

It is noteworthy that in this study a positive correlation ($R^2 = 0.868$) ($P < 0.0001$) was observed between B[a]P content and mutagenic activation ability in the examined meat extract samples. In correspondence with this observation, Rigdon and Neal[23] related B[a]P exposure to tumors of stomach and intestine in mice. In addition, PAH-DNA adducts have been detected at high levels in leukocytes of humans exposed to char-broiled meat[24]. Furthermore, an association between barbecued or grilled meats and tumors of the gastro-intestinal tract has been reported[20]. Additionally, Gunter *et al.*[10] reported an association between high intake of barbecued red meat and colorectal adenoma in a sigmoidoscopy-based study.

Metabolism plays an important role in the conversion of chemical carcinogens into reactive species that damages cellular macromolecules, interferes with signaling pathways and causes cancer[9]. Thus, in order to explain the mechanism of mutagenicity of the examined modelled-heat-treated meat extracts, human hepatoma HepG2 liver cell line was exposed to these extracts.

Interestingly, grilled-meat and pan-fired meat extracts significantly induced phase I enzymes especially, CYP1A1 and CYP1A2 and phase II enzymes especially, NQO1 mRNA expression compared with raw and boiled meat extracts. Consistent with this finding, treatment of the human Caco-2 cell line with PAHs, such as B[*a*]P, chrysene, phenanthrene, benzo[*a*]fluoranthene, dibenzo[*a,b*]pyrene, and pyrene, induced mRNA expression of various xenobiotic metabolizing enzymes, including CYP1A1, CYP1B1, epoxide hydrolase, UGT1A6, and ABC-transport MBR1[17]. However, neither B[a]P nor meat extracts strongly induced UGT1A6 in this study (Fig. 4A). This might be explained by the low concentration of B[a]P in the tested meat extract samples or in the positive control (10 nM, the physiologically relevant concentration in heat-treated meat), which was not enough to induce UGT1A6 mRNA, beside that other extra-hepatic UGT isoforms like UGT1A7 and UGT1A9 also contribute to B[a]P metabolism[17]. CYP1A1, CYP1A2 and NQO1 are the major XMEs responsible for bio-activation of B[a]P to form its mutagenic metabolites. Thus, induction of CYP1A1, CYP1A2 and NQO1 by pan-fried and grilled- meat extracts may contribute to their mutagenic activation ability. Future investigations are still needed to confirm the post-translational and modulatory effects on enzymatic activities of heat-treated meat extracts on XMEs.

In order to reduce the mutagenic potential of heated meat, it is highly recommended to control cooking temperature, time and method. In addition, Kikugawa[15] suggested using reducing sugars to meat before cooking and increasing the water content of meat during cooking or avoiding water loss.

In conclusion, this study declared that heat-treated meat extracts, especially, pan-fried and charcoal grilled-meat extracts induced mutagenic activation of Salmonella typhimurium TA98 in the Ames mutagenicity assay. The analysis of these meat extract samples for their B[a]P content (a true pro-mutagen and pro-carcinogen) indicated that these samples were in the following order: charcoal grilled-meat followed by pan-fried-meat, boiled meat and raw meat extracts, respectively. Induction of XMEs especially CYP1A1, CYP1A2 and NQO1 may contribute to the mutagenic ability of these

extracts. Thus, it is highly advisable to control cooking temperature, time and method in order to reduce cooked-meat mutagens.

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