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RESEARCH ARTICLE

Ultrastructural changes, increased oxidative stress, inflammation, and altered cardiac hypertrophic gene expressions in heart tissues of rats exposed to incense smoke

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Abstract Incense smoke exposure has recently been linked to cardiovascular disease risk, heart rate variability, and endothelial dysfunction. To test the possible underlying mechanisms, oxidative stress, and inflammatory markers, gene expressions of cardiac hypertrophic and xenobiotic-metabolizing enzymes and ultrastructural changes were measured, respectively, using standard, ELISA-based, real-time PCR, and transmission electron microscope procedures in heart tissues of Wistar rats after chronically exposing to Arabian incense. Malondialdehyde, tumor necrosis alpha (TNF)-α, and IL-4 levels were significantly increased, while catalase and glutathione levels were significantly declined in incense smoke-exposed rats. Incense smoke exposure also resulted in a significant increase in atrial natriuretic peptide, brain natriuretic peptide, β-myosin heavy chain, CYP1A1 and CYP1A2 messenger RNAs (mRNAs). Rats exposed to incense smoke displayed marked ultrastructural changes in heart muscle with distinct cardiac hypertrophy, which correlated with the augmented hypertrophic gene expression as well as markers of cardiac damage including creatine kinase-myocardial bound (CK-MB) and lactate dehydrogenase (LDH). Increased oxidative stress, inflammation, altered cardiac hypertrophic gene expression, tissue damage, and architectural changes in the heart may collectively contribute to increased cardiovascular disease risk in individuals exposed to incense smoke. Increased gene expressions of CYP1A1 and CYP1A2 may be instrumental in the incense smoke-induced oxidative stress and inflammation. Thus, incense smoke can be considered as a potential environmental pollutant and its long-term exposure may negatively impact human health.

Keywords Arabian incense · Oxidative stress · Inflammation · Cardiac hypertrophic genes · Cytochrome P450 · Cardiovascular diseases · Cardiac damage · Ultrastructural changes

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Introduction

Incense burning is a common practice in many South East Asian and Middle Eastern nations where incense is burnt as a religious ritual or solely to fragrant homes (Wang 2007; Yeatts et al. 2012). The incense used around the world is typically a combustible mixture of aromatic plant materials and essential oils, which releases fragrant smoke upon burning (Lin et al. 2008). The most common types of Arabian incense used in the Middle Eastern countries are referred to as bakhour and oudh. A wide variety of substances are used to produce

bakhour, such as sandal wood tree resin, agarwood, essential oils, and perfumes, whereas oudh is a homogenously made agar wood from *Aquilaria agallocha*, which develops an aromatic smell due to fungal infection (Wahab and Mostafa 2007; Al-Rawas et al. 2009; Alokail et al. 2011). Upon burning, incense releases particulate matter; gases such as CO, CO_2, NO_2, and SO_2, aldehydes; metallic elements; polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP), naphthalene, and fluoranthene; and volatile organic compounds including benzene, toluene, and xylene (Wang 2007; Yang et al. 2007; Lin et al. 2008; Chuang et al. 2011a). The characteristics of Arabian incense smoke including bakhour and oudh have also been recently described (Cohen et al. 2013). Incense smoke is increasingly being recognized as a potential environmental contaminant based on recent reports describing its association with several chronic diseases. Since inhalation is a mode of exposure, a large number of studies have linked the incense smoke exposure to increased risk of developing respiratory tract carcinomas and other airway diseases (Lin et al. 2008; Friborg et al. 2009; Xie et al. 2014). Arabian incense smoke is also implicated in the development of asthma (Wahab and Mostafa 2007; Al-Rawas et al. 2009). We have earlier shown that long-term Arabian incense smoke exposure contributes to insulin resistance and dyslipidemia in male Wistar rats (Alokail et al. 2011). We also reported that inhalation of smoke from *Boswellia papyrifera* and *Boswellia carterii*, which are essential components of Arabian incense, alters cauda epididymal spermatozoa, spermatogenesis, and sperm parameters in Wistar rats (Ahmed et al. 2013; Ahmed et al. 2014).

A number of environmental contaminants such as cigarette smoke, automobile exhausts, biomass fuel, and air pollution are implicated in the pathogenesis of cardiovascular diseases (CVDs) (Li et al. 2011; Al-Arifi et al. 2012; Lee et al. 2014; Shan et al. 2014). Importantly, these pathological events are reported to be triggered by increased oxidative stress and inflammation (Tousoulis et al. 2008; Lee et al. 2011). Long-term exposure to cigarette smoke has also been shown to cause cardiac tissue damage as evidenced by increased serum levels of lactate dehydrogenase (LDH) and cardiac specific creatine kinase isoform such as creatine kinase-myocardial bound (CK-MB) (Anbarasi et al. 2005; Gokulakrisnan et al. 2011). These experimental pieces of evidence establish the causal link between exposure to environmental toxicants and the risk of developing CVDs.

Cardiac hypertrophy is characterized by an increase in size or thickness of ventricles resulting largely from an increase in individual cardiac myosites. Cardiac hypertrophy reflects a pathological state and is an independent predictor of CVD risk (Frey et al. 2004). Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC) are produced and released by myosites as a compensatory mechanism to positively modulate the cardiac functions against cardiac hypertrophy. Cardiac hypertrophy in response to several cardiotoxins such as cigarette smoke, carbon monoxide, and doxorubicin is associated with the induction of ANP, BNP, and β-MHC genes associating the enhanced expression of ANP, BNP, and β-MHC with the increased CVD risk (Mori et al. 2004; Sanbe et al. 2005; Meurrens et al. 2007; Bye et al. 2008; Richard et al. 2011).

The BaP, a prototype of PAH, one of the major constituents of environmental contaminants, is metabolized by cytochrome P450 (CYP)-dependent monooxygenase system. The members of the CYP family most active in the metabolism of BaP and other chemical carcinogens include CYP1A1, CYP1A2, CYP1B1, and CYP2E1 (Kim et al. 1998; Ioannides and Lewis 2004). Importantly, BaP enhances its own metabolism by inducing the expression of these CYP enzymes (Harrigan et al. 2006; Pushparajah et al. 2008). The metabolism of BaP by the catalytic action of CYP enzymes results in the production of bioactive and unstable intermediary metabolites. These metabolites are cytotoxic and mutagenic and are usually detoxified by the enzymatic action of phase II enzymes (Shimada 2006). Thus, relative expression of CYPs and phase II proteins determines the ratio of activated to detoxified intermediary metabolites. Importantly, studies have found a positive association between induction of CYPs and oxidative stress and inflammation in response to various environmental toxicants, suggesting the possible role of induction of CYPS in the generation of oxidative stress and inflammation (Tousoulis et al. 2008; Tsuji et al. 2011; Raza et al. 2013; Hussain et al. 2014).

Recent studies have found the endothelial dysfunction, heart rate variability, and enhanced CVD risk after exposure to incense smoke (Weber et al. 2011; Huang et al. 2014; Pan et al. 2014). However, the studies investigating the underlying mechanism are lacking. We suspected that increased oxidative stress, inflammation, altered cardiac hypertrophic gene expressions, and cardiac tissue damage and tissue architectural changes might be contributing to pathophysiological events in the increased risk of CVDs in subjects exposed to incense smoke. We further hypothesized that incense smoke might affect the gene expressions of heart xenobiotic-metabolizing enzymes which might be instrumental in the generation of oxidative stress in the heart. Accordingly, in this study, we measured oxidative stress and inflammatory markers, cardiac hypertrophic gene expressions, ultrastructural changes, and the gene expressions of CYP1A1 and CYP1A2 in heart tissue and serum cardiac damage markers in male Wistar rats after chronically exposing to Arabian incense.

Materials and methods

Animals

Male Wistar albino rats (*Rattus norvegicus*) aged 7–8 weeks, weighing 200–210 g, were obtained from the Animal Care Center, College of Pharmacy, King Saud University; Riyadh. The Ethics Committee of the Experimental Animal Care Center approved the study. Animals were housed in a temperature-controlled facility on a 12-h light/dark cycle and had access to water and normal chow diet ad libitum.

Exposure to incense smoke

After 2 weeks of acclimatization, rats were randomly divided into three groups namely control, bakhour, and oudh with each group containing 24 animals. Each group of rats was housed separately from the other to avoid the cross exposure of incense smoke. Two rats at a time from bakhour and oudh groups were subjected to whole-body smoke exposure in inhalation chambers by burning 4 g of respective incense on charcoal daily as described previously (Hussain et al. 2014). Rats from the control group were only exposed to smoke from a burning charcoal. Eight rats from each group were killed after 30, 60, or 90 days from the commencement of the incense smoke exposure. The 60- and 90-day exposure durations were chosen to monitor the effects of incense smoke exposure over longer durations in order to be more realistic to the daily usage of incense smoke in household. The hearts were excised and snap frozen in liquid nitrogen and stored at −80 °C until analyzed. For the analysis of biochemical parameters, tissues were homogenized in 100 mmol KH_2PO_4 buffer containing 1 mmol EDTA (pH 7.4). Homogenates were centrifuged at 12,000×*g* for 30 min at 4 °C, and the clear supernatants were separated and used for the measurement of malondialdehyde (MDA), catalase activity, reduced glutathione (GSH), tumor necrosis alpha (TNF-α), and IL-4 as described below. Total RNA was extracted from the tissues using RNAeasy mini kit (Qiagen, CA, USA) and used for the gene expression analysis of CYP1A1 and CYP1A2 by real-time PCR.

Measurement of MDA

The MDA content was determined according to Draper and Hadley (Draper and Hadley 1990). Briefly, 0.5-ml heart tissue extract supernatant was mixed with 1 ml of trichloroacetic acid solution and centrifuged at 2500*g* for 10 min. The clear supernatant (0.5 ml) of heart tissue homogenate was mixed with 1-ml solution containing 0.67 % thiobarbituric acid (TBA) and incubated for 15 min at 90 °C. The absorbance of the solution was measured at 532 nm, and the concentration of MDA was calculated and expressed as nanomole per milligram protein.

Measurement of catalase activity

The catalase activity was assayed according to the method by Aebi (Aebi 1984) in a final reaction volume of 3 ml containing 0.05 M Tris buffer, 5 mM EDTA (pH 7.0), and 10 mM H_2O in 0.1 M potassium phosphate buffer (pH 7.4). A volume of 50 μl of the supernatant of heart tissue homogenate was added to the above reaction mixture. The rate of change in absorbance per min at 240 nm was recorded. Catalase activity was expressed in terms of micromoles H_2O_2 consumed per minute per milligram of protein.

Measurement of GSH

The measurement of GSH was carried out according to the procedure reported by Owen (Owen 1980). Briefly, 100 μl of clear supernatant of heart tissue homogenate was mixed with 800 μl of 0.3 mM reduced NADPH, 100 μl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), and 10 μl of 50 units/ml GSH reductase. All these reagents were prepared freshly in a phosphate buffer at pH 7.5. The absorbance was measured over a period of 120 s at 412 nm at 30 °C. The GSH level was determined by comparing the rate of change in absorbance of the test solution with that of standard GSH.

Measurement of TNF-α and IL-4

The concentrations of TNF-α and IL-4 were determined by competitive ELISAs following the manufacturer's instructions (My Biosource, CA, USA). Briefly, the heart tissue supernatant and the TNF-α-horseradish peroxidase (HRP) conjugate or IL-4-HRP conjugate were added to the plate precoated with the TNF-α or IL-4 antibodies, respectively. Plates were incubated for 1 h, washed, and incubated with the HRP substrate, 3,3-tetramethylbenzidine. The absorbance was measured at 450 nm using microplate reader. The concentrations of TNF-α and IL-4 were calculated from the standard curve generated by similarly processing the TNF-α and IL-4 standards.

Measurement of CK-MB and LDH

The serum CK-MB and LDH levels in control, bakhour-exposed, or oudh-exposed rats were measured spectrophotometrically following the manufacturer's instructions (United Diagnostics, Dammam, Saudi Arabia). For CK-MB estimation, 40 μl of serum was added to 1 ml of solution containing anti-CK-M antibody and other reactants and incubated for 10 min at room temperature. Absorbance was measured at 340 nm. The LDH levels were measured by adding 50 μl of

serum to 3 ml buffer containing NADH, imidazol, and pyruvate and incubated for 1 min. The absorbance was measured at 340 nm.

Ultrastructural study

Immediately after excision, heart tissues were sliced into small size (1 mm^3) and fixed in 3 % buffered glutaraldehyde for 4 h at 4 °C. Tissue specimens were then postfixed in 1 % osmium tetaroxide (OsO_4) for 90 min. Dehydration of the fixed tissue was performed using ascending grades of ethanol and, then, tissue transferred to resin via propylene oxide. After impregnation with the pure resin, tissue specimens were embedded in the same resin mixture. Ultrathin sections of silver shades (60–70 nm) cut on an ultramicrotome (Leica, UCT) with a diamond knife; sections were then placed on copper grids and stained with uranyl acetate (20 min) and lead citrate (5 min). Stained sections were observed under TEM (JEOL JEM-1011) operating at 80 kV.

Gene expression studies

The relative expressions of ANP, BNP, β-MHC, CYP1A1, and CYP1A2 genes in control and incense smoke-exposed rats were evaluated by real-time PCR. One microgram of total RNA isolated from the heart tissues was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, CA, USA). Real-time PCR was performed on CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) in a 96-well plate format. The reaction mix consisted of cDNA equivalent to 100 ng of total RNA, SYBR green master mix (Kappa Bioscience, MA, USA), and 10 μmol each of forward and reverse primers of GAPDH, ANP, BNP, β-MHC, CYP1A1 or CYP1A2 genes (Table 1). The PCR was carried out with an initial cycle consisting of 94 C for 5 min followed by 40 cycles, each consisting of 94 C, 15 s, and 58 C, 1 min. The GAPDH gene was amplified as an internal control. Each sample was amplified in triplicates. The data were analyzed by ΔΔCt method (Livak and Schmittgen 2001).

Statistical analysis

Data were analyzed using the IBN SPSS version 21.0 (IBM, Armonk, NY, USA). Significance was set at $p<0.05$. Biochemical parameters were expressed as mean±standard deviation (SD). The analysis of variance (ANOVA) was used to compare the various markers in different treatment groups followed by Tukey's honest significant difference (HSD) post hoc test to confirm where the differences occurred between groups.

Table 1 Primer sequences used to amplify target genes by real-time PCR

Target gene	Forward primer (5′–3′)	Reverse primer(5′–3′)
ANP	CGTATACAGTGCGGTG TCCAAC	CCGAGAGCACCTTCTC TCTGAGA
BNP	CAGAAGCTGCTGGAGC TGATAAG	TGTAGGGCCTTGGTCC TTTG
β-MHC	TTGCTGTTATTGCTGCCA TTG	CAAATCGGGAGGAGTT ATCATTC
CYP1A1	CCA TGA CCA GGA ACT ATG GG	TCT GGT GAG CAT CCA GGA CA
CYP1A2	CGCATTGGCTCCACAC CCGTG	ACCGATTCCACCACCT GGTTGACT
GAPDH	TTGGCCGTATCGGACG CCTG	AGCGGAAGGGGCGGAG ATGA

ANP atrial natriuretic peptide, *BNP* brain natriuretic peptide, *β-MHC* beta-myosin heavy chain, *CYP1A1* cytochrome P450 1A1, *GAPDH* glyceraldehyde phosphate dehydrogenase

Results

Oxidative stress markers in heart tissue

The heart MDA, GSH, and catalase activity in control, bakhour smoke-exposed, or oudh smoke-exposed rats are presented in Table 2. Exposure of rats to bakhour for 30, 60, or 90 days led to a significant increase in MDA levels and a significant decrease in catalase activity and GSH levels compared to those in ambient air-exposed control rats. Importantly, the levels of these oxidative stress markers were comparable among the studied time points, indicating that these markers were maximally induced within 30 days of exposure, as no further increase was noted with the sustained exposure for 60 days and at least up to 90 days. Compared to control, oudh exposure resulted in a significant increase in MDA and a significant decrease in GSH levels at the three measured time points. Oudh exposure also significantly truncated the catalase activity at 60-day exposure duration while it had no significant effect at 30 and 90 days of exposure. The bakhour smoke exposure exhibited significantly higher effects on heart MDA levels at 30-day duration than oudh.

Inflammatory markers in heart tissue

The heart TNF-α and IL-4 levels in control, bakhour-exposed, or oudh smoke-exposed rats are provided in Table 3. Rats exposed to bakhour or oudh smoke for 30 days had significantly elevated TNF-α and IL-4 levels as opposed to unexposed controls. The levels of these markers remained unchanged with continued exposure for 60 and 90 days. IL-4 levels in oudh-exposed rats at 60 days and in bakhour-exposed rats at 60 and 90 days were found to be lower than

Table 2 Oxidative stress markers in heart tissues of control, bakhour-exposed, or oudh-exposed rats measured at the indicated times

	30 days			60 days			90 days		
Markers	Control (N=8)	Bakhour (N=8)	Oudh (N=8)	Control (N=8)	Bakhour (N=8)	Oudh (N=8)	Control (N=8)	Bakhour (N=8)	Oudh (N=8)
MDA (nmol/mg)	3.06±0.67	5.16±1.05***	3.60±0.88**,†	4.09±0.98	6.12±1.02***	5.27±0.65*	4.34±0.83	6.42±1.02***	5.59±0.74*
GSH (nmol/mg)	19.14±1.38	16.18±1.76***	16.70±1.13**	19.07±1.28	16.47±1.81**	16.83±1.02*	19.19±1.31	15.99±1.80***	16.83±1.32*
Catalase (pmol/min/mg)	26.25±2.14	22.25±2.55**	24.15±1.57	28.32±2.55	24.19±2.53*	24.42±2.53*	27.89±2.76	23.77±2.86**	24.93±1.58

MDA malondialdehyde, *GSH* reduced glutathione

*p<0.05; **p<0.01; ***p<0.001; †p<0.05, significantly different from bakhour group

Table 3 Inflammatory markers in heart tissues of control, bakhour-exposed, or oudh-exposed rats measured at the indicated times

	30 days			60 days			90 days		
Markers	Control (N=8)	Bakhour (N=8)	Oudh (N=8)	Control (N=8)	Bakhour (N=8)	Oudh (N=8)	Control (N=8)	Bakhour (N=8)	Oudh (N=8)
TNF-α (pg/g)	11.17±1.85	15.56±1.91**	15.18±1.5**	11.49±2.34	15.6±2.61**	15.66±2.87**	11.31±2.23	16.01±1.79**	15.34±3.15**
IL-4 (pg/g)	30.66±2.833	41.8±8.82**	40.79±8.68**	31.47±2.38	43.35±7.1**	39.05±7.49*	30.37±3.93	39.23±7.49*	38.91±6.55*

TNF-α tumor necrosis factor alpha, *IL-4* interleukin-4

*p<0.05; **p<0.01

the levels found in rest of treatments and, however, were significantly higher compared to control.

CK-MB and LDH levels

To assess the impact of incense smoke on cardiac tissue damage, we measured the cardiac markers including CK-MB and LDH levels in control and exposed rats. The serum levels of CK-MB and LDH are provided in Table 4. Compared to unexposed control, rats exposed to bakhour or oudh displayed significantly elevated CK-MB and LDH levels after 30, 60, and 90 days of exposure. At a given time point, bakhour and oudh had identical effects on CK-MB and LDH levels compared to control, except at the 90 days of exposure where oudh had significantly greater effect on CK-MB than bakhour.

Hypertrophic gene expressions in heart tissue

The expressions of ANP, BNP, and β-MHC genes in heart tissues of control and incense smoke-exposed rats are presented in Fig. 1. Compared to control, bakhour-exposed rats demonstrated a 13-, 12.5-, and 13.4-fold increase in ANP messenger RNA (mRNA) levels after 30, 60, and 90 days of exposure, respectively, while oudh-exposed rats had a 11.4-, 11.8-, and 10.9-fold change in ANP mRNA for the said time durations. BNP mRNA levels were increased to 7-, 6.5-, and 6.7-fold in bakhour-exposed rats over control rats subsequent to 30, 60, and 90 days of exposure, respectively. On the other hand, BNP mRNA levels in oudh-exposed rats were 5.5-, 6-, and 6.3-fold higher than those in control rats after exposure for the indicated time. Consistently, rats exposed to bakhour had 11-, 10.5-, and 10.8-fold upregulation in β-MHC mRNA levels over control rats following 30, 60, and 90 days of exposure, while oudh-exposed rats exhibited 9.5-, 9.3-, and 10.2-fold change in β-MHC mRNAs after exposure for identical durations.

Ultrastructural changes in heart muscle

Ultrastructural changes in heart tissues of control and bakhour-exposed, or oudh-exposed rats are presented in Fig. 2. A visible intercalated disc is present with normal mitochondria and muscle fibers in control rats (Fig. 2a). In bakhour-exposed and oudh-exposed groups, the muscle tissue produced hemorrhages, disruption in branching structure with loss of striations, and early necrotic changes in the myocardium. Fewer small clusters of dark-staining cells had thickened muscle bundles that had undergone hypertrophy to five to six times the diameter of the nucleus (Fig. 2b, c). The other

Table 4 Serum cardiac markers in control, bakhour-exposed, or oudh-exposed rats

	30 days			60 days			90 days		
Markers	Control	Bakhour	Oudh	Control	Bakhour	Oudh	Control	Bakhour	Oudh
CK-MB(IU/L)	86.37±12.28	131.75±9.83**	128.0±8.71**	91.0±11.53	158.1±31.3**	169.6±24.8**	102.2±9.91	151.1±15.12**	173.2±21.8**,†
LDH (IU/L)	254.6±13.91	320.6±34.65**	304.0±25.15*	247.3±9.13	293.2±19.21**	286.8±11.58**	257.3±10.21	333.7±33.2**	334.1±36.14**

CK-MB creatine kinase myocardial bound, *LDH* lactate dehydrogenase
*$p<0.01$; **$p<0.001$; †significantly different from bakhour group

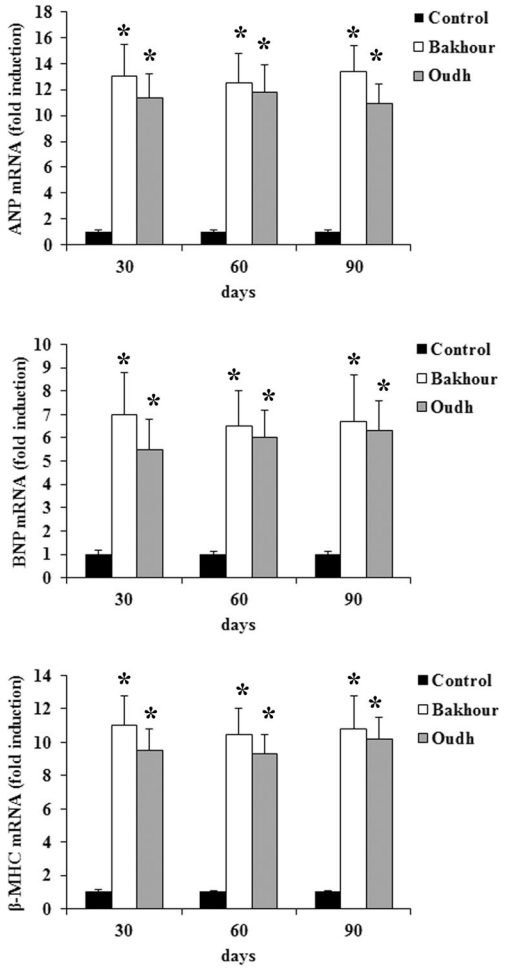

Fig. 1 Fold inductions of ANP, BNP, and β-MHC mRNAs in rat heart. Rats were exposed to bakhour or oudh incense smoke for 30, 60, or 90 days. Total RNA was extracted from the heart tissues of exposed and unexposed control rats at the indicated times. Relative expressions of ANP, BNP, and β-MHC mRNAs in exposed and unexposed control rats were measured by real-time quantitative PCR. The data presented are the mean±SD (N=8), *p<0.001

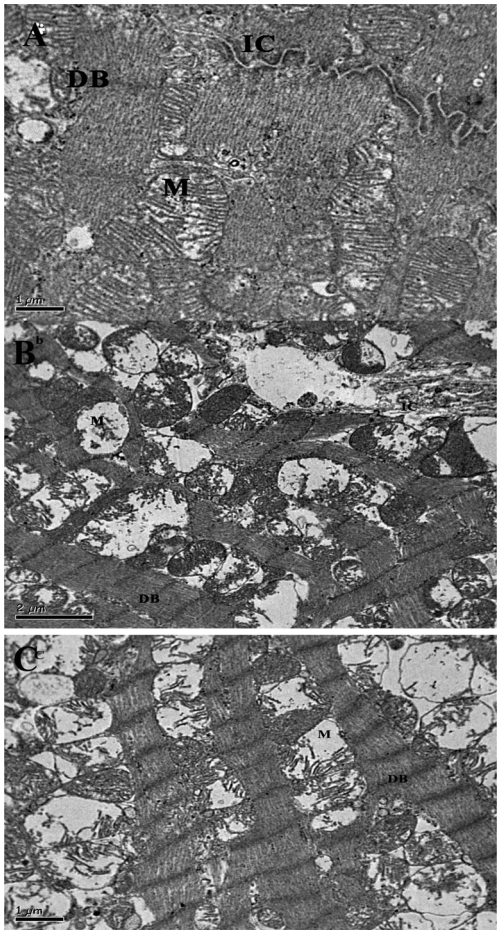

Fig. 2 Ultrastructural changes in rat heart. Rats were exposed to bakhour or oudh incense smoke for 90 days. The hearts were dissected out, and ultrastructural changes in unexposed control (**a**), bakhour-exposed (**b**), or oudh-exposed (**c**) rats were evaluated by transmission electron microscope as described in methodology. Dark bands (*DB*), mitochondria (*M*), and intercalated disc (IC) could be seen within the illustration

organelles including mitochondria and muscle fibers were severely damaged due the toxicity of both the incense types.

Induction of CYP1A1 and CYP1A2 genes in the heart

Extent of CYP1A1 and CYP1A2 gene induction in heart muscle is provided in Fig. 3. Exposure of rats to smoke from bakhour for 30, 60, and 90 days increased heart CYP1A1 gene to 7-, 7.5-, and 7.3-fold, respectively. Likewise, oudh exposure for similar time durations led to 6.5-, 7.2-, and 6.8-fold increase in CYP1A1 mRNA, respectively. The CYP1A2 mRNA demonstrated 5-, 5.5-, and 5.2-fold upregulation following exposure to bakhour for 30, 60, and 90 days, respectively, whereas oudh exposure increased the CYP1A2 mRNA to 5.3-, 4.6-, and 4.8-fold, respectively.

Discussion

Recent studies have indicated increased CVD risk, endothelial dysfunctions, and heart rate variability in incense smoke-exposed individuals. However, the underlying causal mechanisms are unclear. We envisaged that changes to oxidative stress, inflammation, heart architecture, and cardiac hypertrophic gene expressions might collectively contribute to CVD risk in incense smoke-exposed individuals. Accordingly, we studied these parameters in male Wistar rats after a long-term exposure to Arabian incense. Under normal physiological conditions, reactive oxygen species (ROS) produced in the body are negated by the antioxidant defense system, maintaining a healthy balance between oxidant and antioxidant states. However, due to certain pathological or environmental conditions, increased generation of ROS and/or depletion of antioxidants leads to impaired clearance and accumulation of ROS, resulting in oxidative stress. Additionally, continued

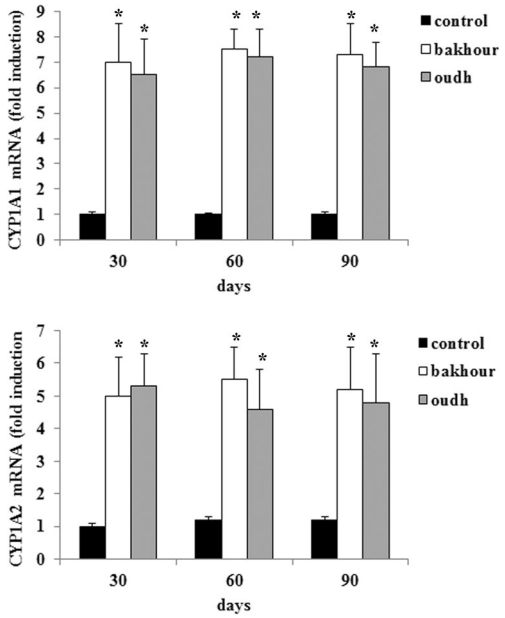

Fig. 3 Fold inductions of CYP1A1 and CYP1A2 mRNAs in rat heart. Rats were exposed to bakhour or oudh incense smoke for 30, 60, or 90 days. Total RNA was extracted from the heart tissues of exposed and unexposed control rats at the indicated times. Relative expressions of CYP1A1 and CYP1A2 mRNAs in exposed and unexposed control rats were measured by real-time quantitative PCR. The data presented are the mean±SD (N=8), *p<0.001

oxidative stress can lead to chronic inflammation, which in turn can increase the risk of chronic diseases (Reuter et al. 2010; Martinon 2010). A number of environmental contaminants such as cigarette smoke, automobile exhausts, biomass fuel, and air pollution are implicated in the pathogenesis of CVDs (Li et al. 2011; Al-Arifi et al. 2012; Lee et al. 2014; Shan et al. 2014). Importantly, these pathological events are reported to be triggered by increased oxidative stress and inflammation, establishing the causal link between exposure to environmental toxicants and the risk of developing CVDs (Tousoulis et al. 2008; Lee et al. 2011). Incense smoke, which is increasingly being recognized as a potential environmental contaminant due to its recently found negative health effects, has not been tested for its ability to induce oxidative stress and inflammation in the heart. In the present study, we found increased MDA and decreased catalase activity and GSH levels in heart tissue of rats after 30 days of incense smoke exposure. Further, the effect of incense smoke sustained with the continued exposure at least up to 90 days, as the levels of oxidative stress markers measured after 60 and 90 days were comparable to those found after 30 days of exposure. This clearly indicates the oxidative stress-inducing capacity of incense smoke in the heart. Moreover, the sustained effects of incense smoke on these markers over a period of 90 days indicate the persistence of oxidative stress with the continued exposure to incense smoke suggesting the adverse health effects of chronic use of incense. Among the incense types used in the study, bakhour has shown relatively higher modulating effects on oxidative stress and inflammatory markers than oudh in the heart. This possibly explains the heterogeneous nature of bakhour as against the homogenous composition of oudh. The reduced catalase and GSH levels possibly reflect the depletion of these antioxidants in the process of continuous quenching of ROS due to their sustained and increased production in the heart. Alternatively, incense smoke may have exerted the direct effect on catalase gene expression and also on gene expression of glutathione reductase, which catalyzes the conversion of oxidized glutathione to its reduced state. We also observed increased levels of inflammatory markers, TNF-α and IL-4, in heart tissue of bakhour or oudh-exposed rats at all the time durations tested. This demonstrates the increased infiltration of the inflammatory mediators in heart tissue, reflecting the direct immunological response to incense smoke or the one mediated by increased oxidative stress (Martinon 2010; Reuter et al. 2010). Our findings are consistent with the previous studies including ours where prooxidant and proinflammatory nature of incense smoke has been reported, albeit in different tissues and in different experimental set ups. For example, we have previously shown that incense smoke increases oxidative stress and inflammation in rat lung and liver tissues (Hussain et al. 2014). The particulate matter of incense smoke is shown to induce significant oxidative stress in human alveolar epithelial cells A459 cells (Chuang et al. 2013). Likewise, incense-derived soot within particulate matter is shown to generate oxidative stress by plasmid scission assay (Chuang et al. 2011b). Increased ROS are also detected in the temple workers exposed to incense smoke pollutants in Taiwan (Ho et al. 2005). Human coronary artery endothelial cells exposed to incense smoke particles exhibited marked increase in IL-6, endothelin, and NO, suggesting the proinflammatory capacity of incense smoke (Lin et al. 2012). Human A459 cells exposed to Arabian incense smoke demonstrated increased inflammatory markers including IL-6 and cyclooxygenase-2 (COX-2) (Cohen et al. 2013). Although studies explaining the inflammatory and oxidative effects of Arabian incense smoke in the heart are lacking, several other environmental contaminants such as cigarette smoke, air pollution, automobile exhausts, and biofuel have all been shown to be capable of inducing oxidative stress and inflammation in the heart (Tousoulis et al. 2008; Das et al. 2012; Miller et al. 2012; Painschab et al. 2013; Raza et al. 2013). Thus, this is the first study to show the prooxidant and proinflammatory effects of incense smoke in the heart and to identify these etiological factors as likely determinants of reported CVD risk in incense smoke-exposed subjects. In this study, we also observed increased serum levels of CK-MB and LDH in incense smoke-exposed rats, indicating cardiac tissue damage. These data are in line with the previous studies where long-term cigarette smoke exposure is shown to increase these cardiac damage markers (Anbarasi et al. 2005; Gokulakrisnan et al. 2011).

Cigarette smoking is known to cause free radical-mediated lipid peroxidation leading to increased membrane permeability and cellular damage in the heart (Cross et al. 1998). Recently, it is shown that cigarette smoke extract adversely affects cell membrane integrity in cardiac stem cells (Sumanasekera et al. 2014). Given the similarities between cigarette smoke and incense smoke compositions, it is likely that the later induced cardiac tissue damage by negatively affecting the membrane integrity resulting in the release of creatine kinase isoforms into the circulation. This possibility also is consistent with the increased oxidative stress found in incense smoke-exposed rats. Corroborating with the increased cardiac damage markers in incense smoke-exposed rats, we also found that incense smoke exposure elicited considerable architectural changes in the heart muscle as evident from the muscle tissue hemorrhages, disruption in branching structure with loss of striations, and early necrotic changes in the myocardium. Fewer small clusters of dark-staining cells had thickened muscle bundles that had undergone hypertrophy to five to six times the diameter of the nucleus. Likewise, mitochondria and muscle fibers were severely damaged due the toxicity of both the incense types. To further understand the extent of cardiac dysfunctions by incense smoke, we examined the changes to the expressions of cardiac hypertrophic genes such as ANP, BNP, and β-MHC. We found markedly increased expressions of all the three studied hypertrophic genes in incense smoke-exposed rats. This clearly points to the development of cardiac hypertrophy in response to incense smoke, as the augmented expression of hypertrophic genes is part of the compensatory mechanism to negate the cardiac hypertrophy which is an independent predictor of CVD risk. Importantly, the upregulated hypertrophic gene expressions positively correlated with the transmission electron microscope data which revealed a significant cardiac hypertrophy in incense smoke-exposed rats. This clearly identifies the hypertrophied muscle as an indicator of an apparent existence of CVD risk. Our findings are consistent with the previous studies where treatment with cardiotoxins such as cigarette smoke, carbon monoxide, and doxorubicin led to a significant hypertrophy with a concomitant increase in ANP, BNP, and β-MHC expressions, thereby relating the cardiac hypertrophy and increased cardiac hypertrophic gene expressions to increased CVD risk (Mori et al. 2004; Sanbe et al. 2005; Meurrens et al. 2007; Bye et al. 2008; Richard et al. 2011). Despite the hypertrophy found in this study, long-term high demand on the heart may also lead to decreased heart size and hypertrophy which may possibly be attributed to differences in nature or magnitude of cardiotoxin exposures. The cardiac tissue degeneration together with increased oxidative stress and inflammation found here is likely an underlying mechanistic event contributing to the reported risk of CVD, endothelial dysfunctions, and heart rate variability in incense smoke-exposed subjects (Weber et al. 2011; Huang et al. 2014; Pan et al. 2014).

To understand the possible mechanism operative in the induction of oxidative stress and inflammation found in incense smoke-exposed rats, we examined the expression of phase I xenobiotic-metabolizing enzymes including CYP1A1 and CYP1A2 which metabolize the BaP, a prototypical PAH and a major constituent of environmental contaminants including of incense smoke (Kim et al. 1998; Ioannides and Lewis 2004; Wang 2007; Chuang et al. 2011a). Importantly, BaP enhances its own metabolism by inducing the expression of these CYP enzymes (Harrigan et al. 2006; Pushparajah et al. 2008). The metabolism of BaP by CYPs results in the generation of reactive and unstable intermediary metabolites which are implicated in the cytotoxicity and genotoxicity (Shimada 2006). Thus, exposure to BaP-containing environmental contaminants contributes to increased production of these intermediary metabolites through the induction of CYPs. Previous studies, including ours, have found a positive association between induction of CYPs and oxidative stress and inflammation in response to various environmental toxicants (Tousoulis et al. 2008; Tsuji et al. 2011; Raza et al. 2013; Hussain et al. 2014). Consistently, induction of CYP1A1 and CYP1A2 in incense smoke-exposed rats correlated with the increased oxidative stress and inflammation in the present study. Therefore, it is likely that increased production and accumulation of reactive intermediates of BaP metabolism due to the increased expression of CYPs contributed to oxidative stress and, in turn, inflammation. Although here, we propose a BaP-mediated induction of CYPs as a mechanism in augmented oxidative stress and inflammation, the possibility of other incense smoke components contributing to oxidative stress cannot be ruled out. The PAHs are high affinity ligands of aryl hydrocarbon receptors (AhRs), which induce the genes containing the xenobiotic response elements. Genes induced by the PAH-AhR pathway includes CYP1A1, CYP1A2, GST, UGT-1, and CYP1B1 (Alexander et al. 1997; Sutter and Greenlee 1992). It is likely that the incense smoke, due to its PAH content, followed similar signaling pathway to induce CYPs in heart tissues.

In conclusion, we proposed here for the first time the increased oxidative stress, inflammation, cardiac tissue damage, and ultrastructural changes to cardiac muscle and augmented expression of cardiac hypertrophic genes as possible pathophysiological events contributing to increased risk of developing CVDs in incense smoke-exposed individuals. We further showed that increased gene expressions of CYP1A1 and CYP1A2 might be an underlying causal mechanism in the incense smoke-induced oxidative stress and inflammation. This study supports the adverse health effects of long-term use of incense smoke.

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References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126

Ahmed M, Al-Daghri N, Alokail MS, Hussain T (2013) Potential changes in rat spermatogenesis and sperm parameters after inhalation of Boswellia papyrifera and Boswellia carterii incense. Int J Environ Res Public Health 10:830–844

Ahmed M, Ali D, Harrath AH, Hussain T, Al-Daghri N, Alokail MS, Aladakatti RH, Ghodesawar MA (2014) Ultrastructural and hormonal changes in rat cauda epididymal spermatozoa induced by Boswellia papyrifera and Boswellia carterii. C R Biol 337:250–257

Alexander DL, Eltom SE, Jefcoate CR (1997) Ah receptor regulation of CYP1B1 expression in primary mouse embryo-derived cells. Cancer Res 57:4498–4506

Al-Arifi MN, Maayah ZH, Alshamrani AA, Korashy HM (2012) Impact of cigarette smoke exposure on the expression of cardiac hypertrophic genes, cytochrome P450 enzymes, and oxidative stress markers in rats. J Toxicol Sci 37:1083–1090

Alokail MS, Al-Daghri NM, Alarifi SA, Draz HM, Hussain T, Yakout SM (2011) Long-term exposure to incense smoke alters metabolism in Wistar albino rats. Cell Biochem Funct 29:96–101

Al-Rawas OA, Al-Maniri AA, Al-Riyami BM (2009) Home exposure to Arabian incense [bakhour] and asthma symptoms in children: a community survey in two regions in Oman. BMC Pulm Med 9:23

Anbarasi K, Vani G, Balakrishna K, Devi CS (2005) Creatine kinase isoenzyme patterns upon chronic exposure to cigarette smoke: protective effect of Bacoside A. Vascul Pharmacol 42:57–61

Bye A, Sørhaug S, Ceci M et al (2008) Carbon monoxide levels experienced by heavy smokers impair aerobic capacity and cardiac contractility and induce pathological hypertrophy. Inhal Toxicol 20: 635–646

Chuang HC, Jones T, Chen Y, Bell J, Wenger J, BeruBe K (2011a) Characterisation of airborne particles and associated organic components produced from incense burning. Anal Bioanal Chem 401: 3095–3102

Chuang HC, Jones TP, Lung SC, BéruBé K (2011b) Soot-driven reactive oxygen species formation from incense burning. Sci Total Environ 409:4781–4787

Chuang HC, Jones T, Chen TT, BéruBé K (2013) Cytotoxic effects of incense particles in relation to oxidative stress, the cell cycle and F-actin assembly. Toxicol Lett 220:229–237

Cohen R, Sexton KG, Yeatts KB (2013) Hazard assessment of United Arab Emirates (UAE) incense smoke. Sci Total Environ 458–460: 176–186

Cross CE, Vliet AV, Eiserich JP (1998) Cigarette smokers and oxidant stress: a continuing mystery. Am J Clin Nutr 67:184–185

Das A, Dey N, Ghosh A, Das S, Chattopadhyay DJ, Chatterjee IB (2012) Molecular and cellular mechanisms of cigarette smoke-induced myocardial injury: prevention by vitamin C. PLoS One 7:e44151

Draper HH, Hadley M (1990) Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 86:421–431

Frey N, Katus HA, Olson EN, Hill JA (2004) Hypertrophy of the heart: a new therapeutic target? Circulation 109:1580–1589

Friborg JT, Yuan JM, Wang R, Koh WP, Lee HP, Yu MC (2009) Incense use and respiratory tract carcinomas: a prospective cohort study. Cancer 113:1676–1684

Gokulakrisnan A, Jayachandran Dare B, Thirunavukkarasu C (2011) Attenuation of the cardiac inflammatory changes and lipid anomalies by (-)-epigallocatechin-gallate in cigarette smoke-exposed rats. Mol Cell Biochem 354:1–10

Harrigan JA, McGarrigle BP, Sutter TR, Olson JR (2006) Tissue specific induction of cytochrome P450 (CYP) 1A1 and 1B1 in rat liver and lung following in vitro (tissue slice) and in vivo exposure to benzo(a)pyrene. Toxicol In Vitro 20:426–438

Ho CK, Tseng WT, Yang CY (2005) Adverse respiratory and irritant health effects in temple workers in Taiwan. J Toxicol Environ Health A 68:1465–1470

Huang YL, Chen HW, Han BC, Liu CW, Chuang HC, Lin LY, Chuang KJ (2014) Personal exposure to household particulate matter, household activities and heart rate variability among housewives. PLoS One 9: e89969

Hussain T, Al-Attas OS, Al-Daghri NM et al (2014) Induction of CYP1A1, CYP1A2, CYP1B1, increased oxidative stress and inflammation in the lung and liver tissues of rats exposed to incense smoke. Mol Cell Biochem 391:127–136

Ioannides C, Lewis DFV (2004) Cytochromes P450 in the bioactivation of chemicals. Curr Top Med Chem 4:1767–1788

Kim JH, Stansbury KH, Walker NJ, Trush MA, Strickland PT, Sutter TR (1998) Metabolism of benzo(a)pyrene and benzo(a)pyrene 7,8-diol by human cytochrome P450 1B1. Carcinogenesis 19:1847–1853

Lee MS, Eum KD, Fang SC, Rodrigues EG, Modest GA, Christiani DC (2014) Oxidative stress and systemic inflammation as modifiers of cardiac autonomic responses to particulate air pollution. Int J Cardiol 176:166–170

Lee S, Park Y, Zuidema MY, Hannink M, Zhang C (2011) Effects of interventions on oxidative stress and inflammation of cardiovascular diseases. World J Cardiol 3:18–24

Li H, Han M, Guo L, Li G, Sang N (2011) Oxidative stress, endothelial dysfunction and inflammatory response in rat heart to NO_2 inhalation exposure. Chemosphere 82:1589–1596

Lin TC, Krishnaswamy G, Chi DS (2008) Incense smoke: clinical, structural and molecular effects on airway disease. Clin Mol Allergy 6:3–10

Lin LY, Lin HY, Chen HW, Su TL, Huang LC, Chuang KJ (2012) Effects of temple particles on inflammation and endothelial cell response. Sci Total Environ 414:68–72

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–8

Martinon F (2010) Signaling by ROS drives inflammasome activation. Eur J Immunol 40:616–619

Meurrens K, Ruf S, Ross G, Schleef R, von Holt K, Schlüter KD (2007) Smoking accelerates the progression of hypertension-induced myocardial hypertrophy to heart failure in spontaneously hypertensive rats. Cardiovasc Res 76:311–322

Miller MR, Shaw CA, Langrish JP (2012) From particles to patients: oxidative stress and the cardiovascular effects of air pollution. Future Cardiol 8:577–602

Mori T, Chen YF, Feng JA, Hayashi T, Oparil S, Perry GJ (2004) Volume overload results in exaggerated cardiac hypertrophy in the atrial natriuretic peptide knockout mouse. Cardiovasc Res 61:771–779

Owen OG (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal Biochem 106:207–212

Painschab MS, Davila-Roman VG, Gilman RH, Vasquez-Villar AD, Pollard SL, Wise RA, Miranda JJ, Checkley W, CRONICAS Cohort Study Group (2013) Chronic exposure to biomass fuel is associated with increased carotid artery intima-media thickness and a higher prevalence of atherosclerotic plaque. Heart 99:984–991

Pan A, Clark ML, Ang LW, Yu MC, Yuan JM, Koh WP (2014) Incense use and cardiovascular mortality among Chinese in Singapore: The Singapore Chinese Health Study. Environ Health Perspect

Pushparajah DS, Umachandran M, Nazir T et al (2008) Up-regulation of CYP1A/B in rat lung and liver, and human liver precision-cut slices by a series of polycyclic aromatic hydrocarbons; association with the Ah locus and importance of molecular size. Toxicol In Vitro 22: 128–145

Raza H, John A, Nemmar A (2013) Short-term effects of nose-only cigarette smoke exposure on glutathione redox homeostasis,

cytochrome P450 1A1/2 and respiratory enzyme activities in mice tissues. Cell Physiol Biochem 31:683–692

Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB (2010) Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Bio Me 49:1603–1616

Richard C, Ghibu S, Delemasure-Chalumeau S, Guilland JC, Des Rosiers C, Zeller M, Cottin Y, Rochette L, Vergely C (2011) Oxidative stress and myocardial gene alterations associated with doxorubicin-induced cardiotoxicity in rats persist for 2 months after treatment cessation. J Pharmacol Exp Ther 339:807–814

Sanbe A, James J, Tuzcu V et al (2005) Transgenic rabbit model for human troponin I-based hypertrophic cardiomyopathy. J Circulation 111:2330–2338

Shan M, Yang X, Ezzati M, Chaturvedi N, Coady E, Hughes A, Shi Y, Yang M, Zhang Y, Baumgartner J (2014) A feasibility study of the association of exposure to biomass smoke with vascular function, inflammation, and cellular aging. Environ Res 135C:165–172

Shimada T (2006) Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. Drug Metab Pharmacokinet 21:257–276

Sumanasekera WK, Tran DM, Sumanasekera TU, Le N, Dao HT, Rokosh GD (2014) Cigarette smoke adversely affects functions and cell membrane integrity in c-kit+cardiac stem cells. Cell Biol Toxicol 30:113–125

Sutter TR, Greenlee WF (1992) Classification of members of the Ah gene battery. Chemosphere 25:223–226

Tousoulis D, Andreou I, Antoniades C, Tentolouris C, Stefanadis C (2008) Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: therapeutic implications for cardiovascular diseases. Atherosclerosis 201:236–247

Tsuji G, Takahara M, Uchi H et al (2011) An environmental contaminant, benzo(a)pyrene, induces oxidative stress-mediated interleukin-8 production in human keratinocytes via the aryl hydrocarbon receptor signaling pathway. J Dermatol Sci 62:42–49

Wahab AA, Mostafa O (2007) Arabian incense exposure among Qatari asthmatic children. A possible risk factor. Saudi Med J 28:476–478

Wang B (2007) Characteristics of emissions of air pollutants from burning of incense in temples, Hong Kong. Sci Total Environ 377:52–60

Weber LP, Al-Dissi A, Marit JS, German TN, Terletski SD (2011) Role of carbon monoxide in impaired endothelial function mediated by acute second-hand tobacco, incense, and candle smoke exposures. Environ. Toxicol Pharmacol 31:453–459

Xie SH, Yu IT, Tse LA, Au JS, Wang F, Lau JS, Zhang B (2014) Domestic incense burning and nasopharyngeal carcinoma: a case-control study in Hong Kong Chinese. Environ Mol Mutagen

Yang TT, Lin TS, Chang M (2007) Characteristics of emissions of volatile organic compounds from smoldering incense. Bull Environ Contam Toxicol 78:308–313

Yeatts KB, El-Sadig M, Leith D et al (2012) Indoor air pollutants and health in the United Arab Emirates. Environ Health Perspect 120: 687–694