Biochemical and Biophysical Research Communications 456 (2015) 92–97

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Secondhand smoke exposure-induced nucleocytoplasmic shuttling of HMGB1 in a rat premature skin aging model

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ARTICLE INFO

Article history:
Received 13 November 2014
Available online 21 November 2014

Keywords:
Secondhand smoke
HMGB1
Premature skin aging
Collagen
Nicotine
Cotinine

ABSTRACT

Secondhand cigarette smoke exposure (SSE) has been linked to carcinogenic, oxidative, and inflammatory reactions. Herein, we investigated whether premature skin aging could be induced by SSE in a rat model, and assessed the cytoplasmic translocation of high mobility group box 1 (HMGB1) protein and collagen loss in skin tissues. Animals were divided into two groups: SSE and controls. Whole body SSE was carried out for 12 weeks. Dorsal skin tissue specimens were harvested for HMGB1 and Mallory's azan staining. Correlations between serum HMGB1 and collagen levels were determined. Rat skin exposed to secondhand smoke lost collagen bundles in the papillary dermis and collagen decreased significantly ($p < 0.05$) compared with control rats. In epidermal keratinocytes, cytoplasmic HMGB1 staining was more diffuse and there were more HMGB1-positive cells after four weeks in SSE compared to control rats. A negative correlation between HMGB1 serum and collagen levels ($r = -0.631$, $p = 0.28$) was also observed. Therefore, cytoplasmic HMGB1 expression in skin tissues might be associated with skin collagen loss upon the initiation of SSE. Additionally, long-term SSE might affect the appearance of the skin, or could accelerate the skin aging process.

1. Introduction

Tobacco smoke from secondhand smoke contains almost 4000 chemicals, including 50 known carcinogens such as nicotine, benzene, formaldehyde, and hydrazine [1]. Secondhand smoke exposure (SSE) impairs the function of many organs. Long-term health risks include the impairment of the growth and development of children and a higher likelihood of developing cancer [2]. Chronic smoking has been shown to alter vasodilatory capacity in the cutaneous microcirculation [3]. The specific damaging effects on the skin can result in poor wound healing, squamous cell carcinoma, melanoma, and premature skin aging [4]. Additionally, tobacco smoking has been identified as an important factor in premature skin aging based on epidemiological studies [5,6].

Collagens are important proteins for the skin, as they are essential for the structure and function of the dermal extracellular matrix. Thinner and wrinkled skin—typical signs of normal aging—result from reduced collagen [7]. Protein glycation contributes to skin aging as it degrades existing collagen molecules by crosslinking [8]. Topical or intracutaneous injection of tobacco smoke extract can induce premature skin aging [9]. Collagen levels are significantly reduced via extracellular matrix-associated members of the matrix metalloproteinase (MMP) gene family. The expression of *MMP-1* and *MMP-3* mRNA can be induced in a dose-dependent manner in cultured skin fibroblasts that are treated with tobacco smoke extract [9,10]. Additionally, cigarette smoke extract can provoke reactive oxygen species (ROS) release, which can induce nucleic acid oxidation. These activities drive collagen induction and elastic fiber degradation [11].

High mobility group box 1 (HMGB1) protein is a nuclear protein that was identified as a potent pro-inflammatory mediator that can translocate from the nucleus to the cytoplasm and extracellular space [12]. HMGB1 can be passively released by necrotic or damaged cells [13] and can be detected in serum samples as a biomarker [14,15]. SSE can lead to HMGB1 release from injured cells into blood circulation [16], and cytoplasmic translocation has been detected in lung tissues from SSE-exposed rats [17]. HMGB1

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http://dx.doi.org/10.1016/j.bbrc.2014.11.040
0006-291X/

release is increased in the skin of SLE patients compared to healthy controls, and HMGB1 release contributes to the development of inflammatory skin lesions [18]. Ultraviolet (UV) irradiation induces an inflammatory skin response that is initiated by HMGB1 release from UV-damaged keratinocytes [19]. Despite the importance of HMGB1 in inflammatory process and wound repair, very few studies have examined its role in aging and no study has evaluated its expression in the skin following cigarette smoke exposure. This study aimed to investigate the effect of SSE on collagen formation and HMGB1 expression in prematurely aged skin in a rat model.

2. Materials and methods

2.1. Detection of nicotine content in cigarette smoke condensates

Cigarettes smoke condensates were dissolved in ethanol and nicotine content was detected by reverse phase high-performance liquid chromatography (HPLC), as described previously [20,21].

2.2. Animals and secondhand smoke exposure (SSE)

Male Wistar rats that weighed 250–300 g were purchased from the National Laboratory Animal Center (Mahidol University, Nakhon Pathom, Thailand). All rats were housed under a 12-h light/dark cycle at a controlled temperature of 22 ± 2 °C with food and water provided *ad libitum*. All animal protocols were approved by the Committee for Laboratory Animals Use of Mahidol University.

The experiment was designed to include 24 rats, which were allocated into one of two groups: control and SSE rats. Exposure to secondhand smoke was conducted for 3 months using a smoking machine, and the equivalent of 10 cigarettes were provided 5 days per week (Monday through Friday) at 8 am and 5 pm. Treatments were randomized, and the investigators were blinded to the specific treatment.

2.3. Skin specimen preparation for immunohistochemical analyses

Rat skin sections were stained with anti-HMGB1 rabbit polyclonal antibody (Shino-Test Corporation) according to the protocol described in our previous study [22]. Mallory's azan stain was used to visualize collagen fibers. Histological sections were imaged at 40× magnification, and digital images were analyzed using Image Pro Plus 6.0 software to measure epithelial and dermal layer thickness. In each section, three separate sites were measured by two trained examiners who were blinded to the group being analyzed.

2.4. Collagen quantification

The Sircol collagen assay (Biocolor Ltd., Belfast, United Kingdom) was performed as described previously [23]. Samples were analyzed using a microplate reader and the absorbance was determined at 540 nm.

2.5. Preparation of nuclear and cytosolic fractions

Skin tissues were extracted for the analysis of HMGB1 expression in the nucleus and cytoplasm using a compartmental protein extraction kit (Chemicon, Billerica, MA, USA) according to the manufacturer's instructions.

2.6. Western blot analysis

All samples were subjected to 12% SDS–polyacrylamide gel electrophoresis, as previously described [24].

2.7. Measurements of cotinine and HMGB1 levels by ELISA

The levels of cotinine sera were measured by ELISA (DRG Instruments, Marburg, Germany). HMGB1 levels in serum were evaluated by ELISA (Shino-Test Corporation).

2.8. Statistical evaluations

Differences in HMGB1 levels in rat sera were determined by one-way analysis of variance followed by the Bonferroni *post hoc* test for multiple comparisons. Differences between any two groups were analyzed using unpaired Student's *t*-test or the Mann–Whitney test as appropriate. The Pearson's product-moment correlation coefficient was used for correlation analysis. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Nicotine content in cigarette smoke condensates

The resulting chromatogram (at 259 nm) is shown in Fig. 1A. The chromatogram included a peak within the retention time of 5.79 min that we identified to be nicotine. The concentration of nicotine in the cigarette smoke condensates was ~0.06 μg per cigarette.

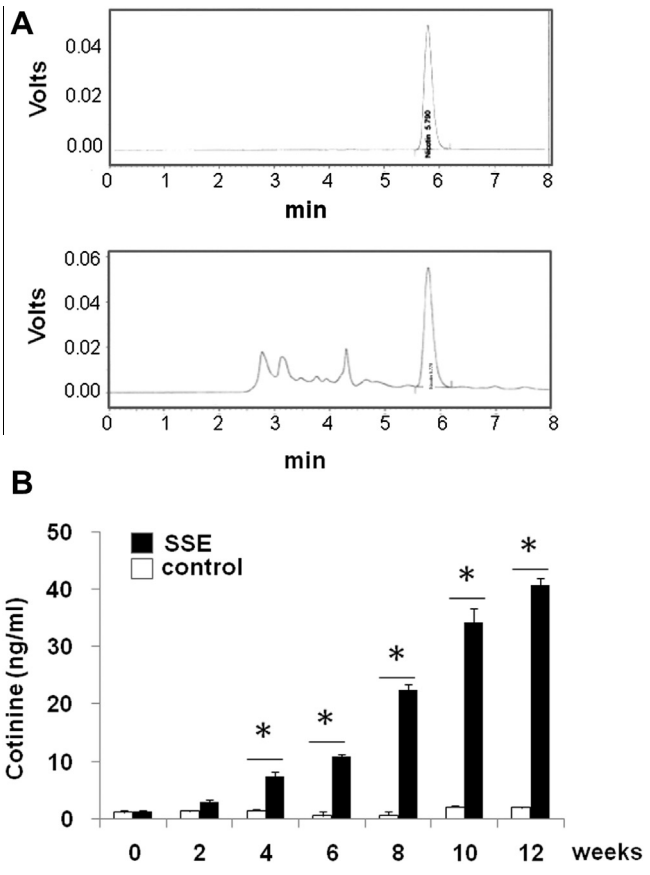

Fig. 1. Nicotine levels in cigarette smoke condensates and cotinine levels in the serum of rats. (A) HPLC analysis of nicotine content. The chromatogram was based on readings at 259 nm. A peak was detected with a retention time of 5.79 min. A representative chromatogram of triplicate experiments is shown. (B) Cotinine levels in week 12 SSE rats were analyzed by ELISA. Data represent means ± SD; $n = 8$; $^{*}p < 0.001$ vs. the control group).

3.2. Body weights and serum cotinine levels

Rat body weights were monitored weekly. After 6–12 weeks of SSE, the average body weight of the SSE-exposed rats was significantly lower than that of the control group rats ($p < 0.001$; Table 1). Cotinine levels in the SSE group were significantly higher than those in the control group from week 4 until the end of the experiment (Fig. 1B; $p < 0.001$).

3.3. Skin collagen and measurements of epidermal and dermal layer thickness

Rat skin sections were stained with Mallory's azan and Mayer's hematoxylin (Fig. 2A). Skin with histologically confirmed lesions was characterized by the loss of collagen staining, which could be observed in the upper dermis in rats following 12 weeks of SSE (a–c) compared with control rats (d–f). Collagen was organized into dense and discrete bundles in both the upper and papillary dermis, and formed thicker bundles in the deeper or reticular dermis in the skin of control rats. In SSE rat skin, a thinner mean epithelial thickness was mostly a consequence of the loss of the spinous layer in the skin of SSE rats (37.43 ± 6.07 μm) compared to control rats (46.20 ± 2.09 μm; Fig. 2B). However, there was no statistically significant difference between the mean dermal layer thickness in the skin of SSE-treated rats (1629.66 ± 0.03 μm) and control group rats (1621.93 ± 3.20 μm; $p > 0.05$). Furthermore, we quantified the amount of collagen in skin tissues (Fig. 2C). The average collagen level in the SSE group was significantly lower than that in the control group (0.03 ± 0.02 vs. 0.10 ± 0.04 μg/mg; $p = 0.001$). These data indicate that SSE can reduce collagen bundle content.

3.4. Nucleocytoplasmic shuttling of HMGB1 from premature skin aging tissues to the serum

Our findings demonstrated the clear translocation of nuclear HMGB1 to the cytoplasm in tissues from the skin of SSE-exposed rats, whereas HMGB1 was mainly present in the nuclei of tissues from control rats (Fig. 3A). We confirmed whether increased expression of HMGB1 in the cytosol was visible in SSE skin tissues *in situ*. Skin tissues were obtained and were stained with anti-HMGB1 antibody (Fig. 3B). We found that HMGB1 was localized in nuclei of epidermal keratinocytes and the endothelial-like cell lining at week 0 in control (a and b) and SSE-treated (c and d) rats. A similar staining pattern could be observed at 4 and 12 weeks in the control group rats (data not shown). After 4 weeks of SSE exposure, a broad distribution of the number of HMGB1-positive cells and strong staining in the cytoplasms of epidermal keratinocytes in SSE rats could be observed (e and f). However, there appeared to be less epidermal staining at 12 weeks (g and h). Additionally,

Table 1
A comparison of rat body weights between study groups.

Week	Rat body weights (g)		
	SSE	Controls	*p*-Value
0	277.4 ± 8.85	283.4 ± 9.27	0.12
2	300.0 ± 3.30	304.0 ± 6.59	0.06
4	355.1 ± 23.34	348.6 ± 10.38	0.39
6	364.4 ± 18.07*	396.1 ± 6.21	<0.001
8	379.6 ± 18.34*	436.5 ± 7.81	<0.001
10	402.6 ± 21.08*	463.0 ± 10.05	<0.001
12	423.8 ± 25.12*	476.8 ± 9.18	<0.001

SSE, secondhand smoke exposed group. Values indicate means ± SD ($n = 12$). Statistically significant differences were detected by Student's *t*-test;
* $p < 0.001$ vs. the control group.

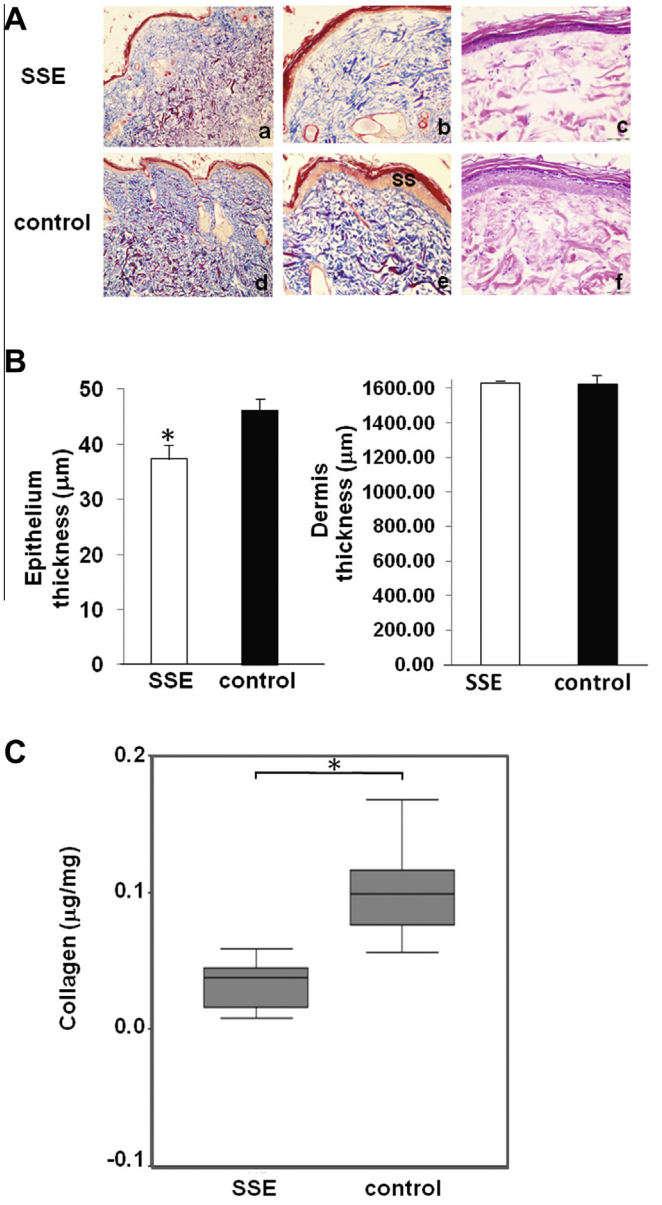

Fig. 2. Reductions in collagen fibers and epithelium thickness in SSE rat skin tissues. (A) Representative skin sections show the collagen bundles of SSE (a–c) and control (d–f) rats. Sections were stained with Mallory's azan (a, b, d and e) or hematoxylin and eosin (c and f). Note that blue indicates collagen bundle stained by Mallory's azan stain. SS, stratum spinosum. All images were taken at 20× magnification, except for (a and d) that were taken at 10× magnification; $n = 5$. (B) The average skin epithelial and dermal layer thickness was measured for SSE and control rat skin tissue. Data represent the means ± SD of three images ($n = 10$, $^*p < 0.05$). (C) The box plot depicts the collagen levels that differed significantly between the SSE and control groups. Box plots represent the median levels and the 25th and 75th percentiles of the observed data; whiskers represent the 5th and 95th percentiles in each group. Data were generated from the SSE ($n = 9$) and control ($n = 7$) groups; $^*p = 0.001$ vs. the control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

no positive staining of the isotype control IgG was detected in tissues from either group (data not shown). To determine whether the reduction in cellular HMGB1 levels was caused by the release of protein after SSE, HMGB1 levels were measured in the serum (Fig. 3C). Serum levels of HMGB1 were significantly higher in SSE rats after 6 weeks compared to week 2 or 4 SSE rats ($p < 0.001$). After week 6, levels of HMGB1 were significantly higher in the SSE group than in control rats ($p < 0.001$). Our results indicated

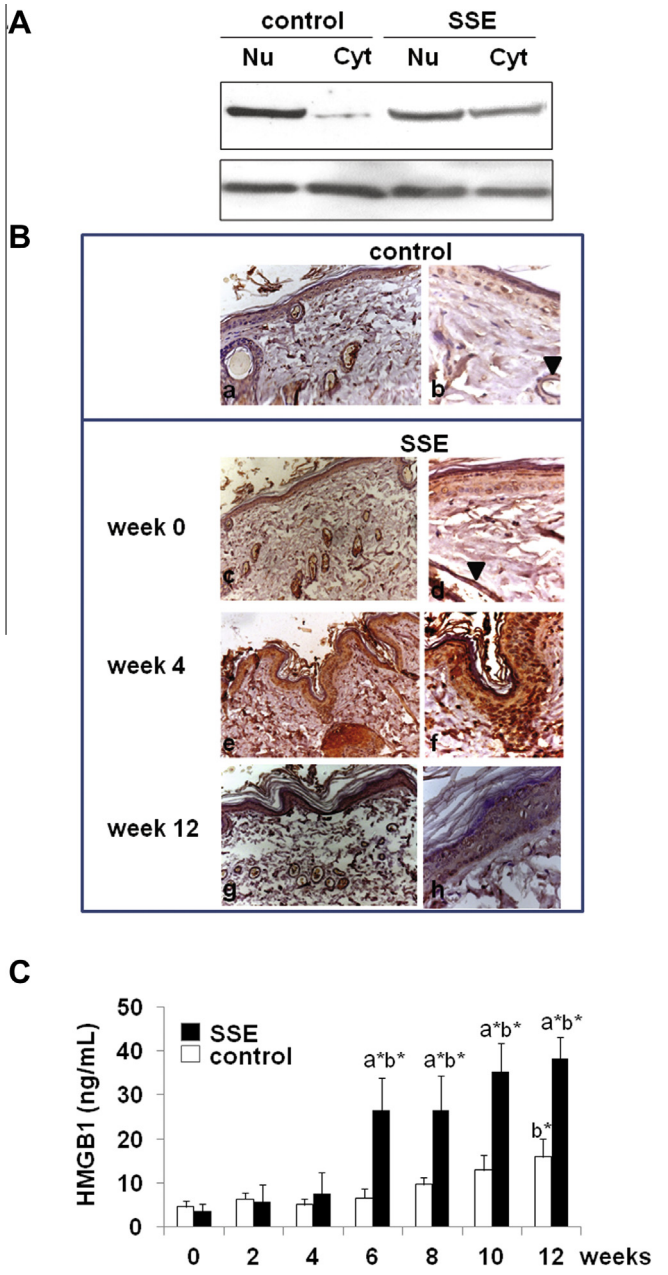

Fig. 3. HMGB1 protein up-regulation in cytoplasmic extracts and serum of SSE rat. (A) Nuclear (Nu) and cytoplasmic (Cyt) fractions were assayed for HMGB1 levels in SSE and control rats and were analyzed by Western blotting. β-Actin was used as a loading control. (B) Immunohistochemical analysis of HMGB1 expression in the SSE rat tissues after 0, 4, or 12 weeks and in control rats. Arrowhead, blood vessel. Original magnifications: 100× (left), 400× (right). (C) ELISA data show increased serum HMGB1 levels in SSE rats. Data are means ± SD ($n = 12$ rats per group). [a*]Significant differences were detected between the SSE and control groups. [b*]Significant differences were detected between weeks 6–12 and week 0 rats; $^{*}p < 0.001$.

that HMGB1 translocated from the nuclei to the cytoplasm, and was then secreted from skin tissue into circulating blood.

3.5. Correlations between HMGB1 and collagen levels

The levels of HMGB1 within the SSE group at the end of study were 27.11 ± 12.42 ng/mL, and the levels of collagen within the skin tissues were 0.70 ± 0.45 μg/mg. A negative correlation between HMGB1 serum levels and the volume of collagen ($r = -0.631$, $p = 0.028$) was detected (Fig. 4).

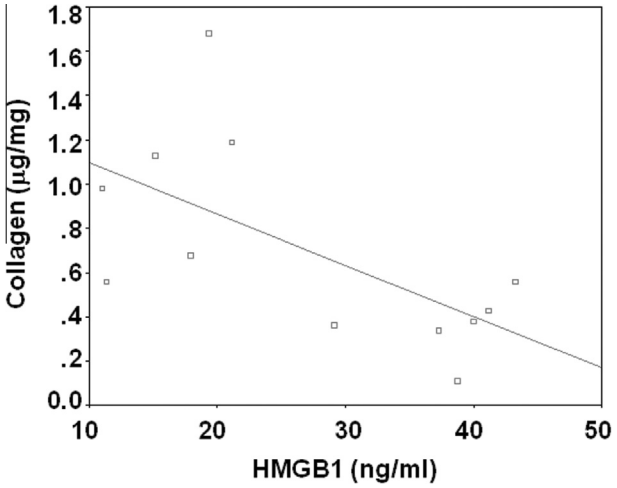

Fig. 4. Correlation of serum HMGB1 and collagen levels in premature aging skin of rats. Pearson's r was used to show the correlation between the levels of serum HMGB1 and skin collagen at week 12 ($n = 12$).

4. Discussion

To the best of our knowledge, this study represents the first attempt to examine the effects of secondhand cigarette smoke on both premature skin aging and HMGB1 cytotranslocation. Our findings demonstrated that the skin of SSE rats lost collagen bundles in the papillary dermis, and collagen was decreased significantly the skin tissues, as assessed by immunostaining and the Sircol assay, respectively. Cytoplasmic HMGB1 immunostaining, protein expression, and serum levels were all higher in SSE than in control rat tissues. Furthermore, a negative correlation between HMGB1 serum levels and collagen levels in SSE rats was detected.

Many factors, both extrinsic and intrinsic, can contribute to skin aging [11]. Environmental factors are the result of daily exposure to trillions of free radicals from extrinsic environmental sources, such as ultraviolet rays, pollution, harsh weather, external stress, and smoke [25]. Although nicotine can be directly absorbed through the skin [26], the whole bodies of rats were exposed to cigarette smoke, so nicotine might have entered the body through the skin and by inhalation in our study. Cotinine is a metabolite of nicotine [27] that can induce HMGB1 release [16]. We found that SSE markedly increased serum cotinine levels in rats after 4 weeks. Additionally, rat body weights decreased after 6 weeks of SSE, whereas there was no change in control group rats. Nicotine-mediated nicotinic receptor signaling can suppress appetite and reduce food intake [28]. We showed that this rat model could also be affected by cigarette smoke in our experimental conditions.

Collagen loss in cigarette smokers occurs directly via changes in vasodilatory capacity in the cutaneous microcirculation [3]. Previous studies showed that cigarette smoke extract can trigger reactive oxygen species (ROS)-dependent nucleic acid oxidation-mediated induction of collagen and elastic fiber degradation [11]. Specifically, nicotine can induce ROS formation [29]. Additionally, oxidative stress can induce the release of active HMGB1 [30]. SSE might directly affect the blood supply to rat skin, or act indirectly via ROS and HMGB1 stimulation to promote premature skin aging. Further studies using *in vitro* assays should be employed to determine whether cigarette smoke content (i.e. nicotine or cotinine) can interfere with collagen degradation.

The receptor for advanced glycation end products (RAGE) acts as a receptor for multiple ligands, including HMGB1, and the RAGE–nuclear factor-kappaB (NF-κB) pathway can be initiated by oxidative stress [31]. ROS is thought to play an important role in

cutaneous pathologies. Interestingly, our data regarding the cyto-translocalization of HMGB1, a RAGE ligand that is released into the serum following SSE, might support a recent study showed that the overexpression of RAGE can contribute to cigarette smoke-induced translocation of p65 (the NF-κB family), thereby promoting tissue injury and inflammation [32].

HMGB1 acts as an "early warning signal" type of cytokine [33] that can be passively released by necrotic or damaged cells. Reduced nuclear HMGB1 staining is commonly interpreted to be a sign of HMGB1 release *in vivo* in models of injury [34]. In this study, we investigated HMGB1 protein translocation from the nucleus to the cytoplasm in skin cells and also measured its release in the serum. Our findings showed that the robust translocation of HMGB1 within skin tissues occurred after exposure to cigarette smoke for 4 weeks, and we could observe reduced HMGB1 staining by week 12. However, serum HMGB1 levels were elevated after 6 weeks of SSE. Our findings are in accordance with those of a previous study [34] that showed that reduced HMGB1 staining was likely a consequence of HMGB1 release into the serum.

Our study used cigarettes that contained 1.1 ± 0.3 mg nicotine according analysis conducted by the Research and Development Department, Thailand Tobacco Monopoly. Therefore, the use of 10 cigarettes daily represents exposure to ~11 mg nicotine per day. However, we measured nicotine content and found that the level was 0.6 μg in the smoke condensate from 10 cigarettes. Therefore, assuming an average rat body weight of 0.4 kg, the average amount of daily nicotine consumption for a rat is 15 μg/kg bodyweight, which represents the total nicotine dose that has been used in previous rat studies [35].

Although most studies have focused on the influence of the direct application of cigarette smoke extract on skin pathology *in vivo* [9,36] and *in vitro* [32], there is scarce experimental data on the influence of secondhand cigarette smoke on premature skin aging after chronic exposure. From this perspective, we conclude that SSE can affect collagen formation and induce HMGB1 release, which can be used as a marker of the burden of premature skin aging in an individual. These data might indicate that SSE is associated with a greater biological risk (as a surrogate for environmental smoke) than is currently recognized.

Acknowledgments

This study was supported by the Thai Health Promotion Foundation (Grant number 52-00-0882-07/2553) and Mae Fah Luang University, Thailand.

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