Smoking State Determined by Cotinine and Arterial Stiffness

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Background: Exposure to active and passive smoking is associated with arterial stiffness and cotinine is an objective biochemical indicator to classify smoking state. The aim of the study was to survey participant smoking condition with cotinine in North China and discuss its destructive effect on arterial stiffness.

Methods and Results: Nine hundred and six people were recruited in this study and were classified into non-smokers, passive smokers and active smokers according to the cotinine level. Brachial-ankle pulse wave velocity (baPWV) was measured using an automatic device. Active smokers vs non-smokers showed a significantly higher baPWV ($P=0.023$) after adjusting for covariates. Participants who smoked longer than 10 years had a higher baPWV than non-smokers ($P=0.029$) although no significant difference existed between those who had a smoking history of less than 10 years and those who had never smoked. In less developed areas, the active and passive smoking rates were higher than that in Beijing ($P<0.001$) while active smokers vs non-smokers had a higher baPWV ($P=0.017$).

Conclusions: Active smokers, as confirmed by the cotinine level have worse arterial stiffness, and long-term smoking can cause marked arterial stiffness. Smoking exposure might bring about more serious effects on arterial function in patients from poor areas than in modernized cities. (Circ J 2009; 73: 1537–1542)

Key Words: Arterial stiffness; Brachial-ankle pulse wave velocity; Cotinine; Smoking state

Cigarette smoking is a major preventable risk factor for death in China! Several studies have demonstrated that exposure to active and passive smoking is associated with arterial stiffness,^{2–7} which is an important pathological state of vascular damage and is closely related to atherosclerotic cardiovascular disease.⁸ Measurement of pulse wave velocity (PWV) is one of the most representative non-invasive techniques for assessing arterial stiffness. Simple methods to measure brachial-ankle PWV (baPWV) by recording the distensibility of the brachial and tibial arteries in just 5 min had been available for several years^{9,10} and former studies have proven its favorable correlation with central PWV, as examined by invasive methods,¹¹ and with other traditional risk factors for cardiovascular disease.¹² In previous studies to evaluate the harmful effect of cigarette exposure on arterial stiffness, a confidential questionnaire about smoking state was always administered during a specific interview by investigators.^{13,14} However, the information about smoking based on the questionnaire does not always indicate objective conditions.^{15–19} Our aim was to investigate the smoking condition of patients from in North China, using cotinine, a sensitive and specific marker of active and passive exposure to tobacco,^{18–21} and discuss its impact on arterial stiffness.

Methods

Study Population

Between January and August 2008, 906 people, aged 20–70 years, were recruited for this study in Beijing and Julu, a small town 400 km south of Beijing. These subjects included inpatients and outpatients as well as volunteers who underwent health-screening examinations in the medical examination centers. For the entire population, the exclusion criteria included patients who had a history of: (1) percutaneous coronary intervention and/or coronary artery bypass graft treatment; (2) aortic valve heart disease; (3) aortic aneurysm; (4) serious myocardial dysfunction with an ejection fraction of $<30\%$; and (5) peripheral arteriosclerosis obliterans with an ankle-brachial index (ABI) of <0.9 .

Self-reported smokers were defined if the subjects confessed to smoking cigarettes daily for at least 6 months and were further categorized as current and former smokers. Former smokers were included in this study if they had given up smoking for longer than 10 years and were ascribed as non-smokers during analysis. Thirty-seven people were excluded from the study because they had less than 10 years of smoking cessation.

All subjects gave written informed consent, and the study protocol was approved by the Beijing Army General Hospital. Fasting venous blood samples were drawn, and total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), triglycerides (TG), and serum glucose were measured by using standard methods.

Measurement of Cotinine

The assay was performed using a commercial 96 well (STC Cotinine Micro-plate EIA, Cat.no.CT086D; Calbiotech Inc, Spring Valley, CA, USA). All reagents were brought to room temperature ($18–26^{\circ}\text{C}$) before use. The procedure used was as follows: add $10\mu\text{l}$ of standards and serum to

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Table 1. Characteristics of the Study Cohort According to Smoking Status

	Non-smokers (n=617)	Passive smokers (n=83)	Active smokers (n=206)	P value		
				A	B	C
Age (years)	47.2±12.3	42.7±13.4	46.5±11.3	0.002	0.016	0.524
BMI (kg/m ²)	24.4±3.4	24.5±3.4	25.5±3.3	0.738	0.028	<0.001
HR (beats/min)	70.6±9.7	70.9±9.5	71.6±10.8	0.821	0.565	0.209
SBP (mmHg)	128±20	124±15	132±17	0.037	0.001	0.026
DBP (mmHg)	78±12	78±11	82±11	0.671	0.002	<0.001
ABI	1.10±0.08	1.10±0.07	1.11±0.08	0.847	0.650	0.312
CIMT (mm)	0.71±0.20	0.68±0.19	0.75±0.21	0.133	0.003	0.009
TC (mmol/L)	4.80±0.93	4.58±0.76	4.80±0.99	0.038	0.060	0.986
LDL-C (mmol/L)	2.75±0.72	2.62±0.71	2.77±0.80	0.141	0.119	0.706
HDL-C (mmol/L)	1.34±0.28	1.32±0.26	1.30±0.26	0.420	0.628	0.051
TG (mmol/L)	1.59±1.03	1.83±1.32	1.98±1.37	0.607	0.313	<0.001
Glucose (mmol/L)	5.27±1.22	5.17±0.87	5.32±1.35	0.511	0.039	0.017
Hypertension (%)	233 (37.8)	23 (27.7)	102 (49.5)	0.089	0.001	0.004
ACEI/ARB (%)	35 (15.0)	3 (13.0)	15 (14.7)	0.796	0.838	0.940
CCB (%)	58 (24.9)	7 (30.4)	23 (22.5)	0.809	0.427	0.338
No treatment (%)	83 (35.6)	11 (47.8)	46 (45.1)	0.267	0.821	0.113
Diabetes (%)	64 (10.4)	6 (7.2)	23 (11.2)	0.441	0.390	0.794
Any treatment (%)	24 (37.5)	3 (50.0)	9 (39.1)	0.670	0.669	0.891

A, Passive smokers vs Non-smokers; B, Active smokers vs Passive smokers; C, Active smokers vs Non-smokers; BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; ABI, ankle-brachial index; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; ACEI/ARB, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker; CCB, calcium channel blocker.

each well, and then 100 μ l of the enzyme conjugate to each well. Tap the sides of the plate holder to ensure proper mixing. Incubate for 60 min at room temperature (18–26°C) in the dark, after the addition of an enzyme conjugate to the last well. Then wash the wells 6 times with 300 μ l distilled water using a plate washer taking care not to cross-contaminate wells. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. Add 100 μ l of substrate reagent to each well and tap the sides of the plate holder to ensure proper mixing. And then incubate for 30 min at room temperature in the dark. Add 100 μ l of stop solution to each well to change the blue color to yellow. The cotinine concentration in each well was then quantified by measuring the light absorbance at wavelengths of 450 nm and comparing them with a standard curve; this technique is sensitive to 1 ng/ml.

The participants who were regarded as non-smokers with no marked passive cigarette exposure had to have a serum cotinine level lower than 1 ng/ml. Marked passive smoking was diagnosed when the level of serum cotinine ranged between 1 and 15 ng/ml. The subjects were classified as active smokers when their cotinine levels exceeded 15 ng/ml^{15,20,22}

Measurement of baPWV

The baPWV was measured using an automatic device (Model BP-203PRE, Colin, Inc, Komaki, Japan). baPWV, systolic blood pressure (SBP), diastolic blood pressure (DBP), electrocardiogram, and heart sounds were recorded simultaneously. After a 5 min rest in the supine position, pressure waveforms of the brachial and tibial arteries were recorded with cuffs wrapped on both brachia and ankles, the electrocardiogram was recorded using electrodes placed on both wrists, and a microphone for detecting heart sounds was placed on the left edge of the sternum. The pressure waveforms obtained by the cuff were derived from a plethysmographic sensor that determines volume pulse form and an oscillometric sensor that measures blood pressure. Volume waveforms at the brachial and ankles were stored for a sampling time of 10 s with automatic gain analysis and

quality adjustment. The time interval between the brachial and ankle (Tba) was the time interval between the initial rise in the brachial and tibial pressure waveforms. The distance between sampling points of baPWV was calculated automatically based on body height. The length from the aortic valve to the ankle (La) was estimated using the following equation: $La = 0.8129 \times \text{height} + 12.328$ (all values for length and height are in cm). The length from the aortic valve to the brachial (Lb) was estimated using the following equation: $Lb = 0.2195 \times \text{height} - 2.074$. The baPWV was estimated using the following equation: $\text{baPWV} = (La - Lb) / Tba$. Bilateral baPWV and brachial and ankle blood pressures were measured automatically and simultaneously by this device. We used the mean value of the bilateral baPWV and only the left brachial blood pressure in our analyses.

Statistics Analysis

Continuous variables were presented as mean $\pm$ standard deviation, and categorical variables were presented as percentages. A P value less than 0.05 was considered significant. A one-way ANOVA was used to compare the clinical characteristics of studied subjects among various smoking groups. Chi-square tests were used to compare proportions across groups. A partial correlation coefficient was reported for the association between cotinine and baPWV, which controlled for age, heart rate (HR), DBP, serum glucose, body mass index (BMI), TG, and gender. They were also added in the analysis of covariance (ANCOVA) model as covariates when comparing the differences of baPWV among different groups. The statistical calculations were performed with the SPSS 13.0 program.

Results

Clinical Characteristics

According to the cotinine level, 617 subjects were classified as non-smokers, 83 were diagnosed as obviously passive smokers, and 206 were diagnosed as active smokers. The median levels of cotinine for passive smokers and active

Table 2. Comparison of baPWV Among Different Smoking Status and Different Smoking Duration

Smoking status	Unadjusted analysis			Adjusted analysis [#]		
	baPWV (cm/s)	Difference (95%CI)	P value	baPWV (cm/s)	Difference (95%CI)	P value
Non-smokers	1,407			1,396		
Passive smokers	1,346	-61.3 (-128.5~6.0)	0.740	1,403	6.4 (-61.0~73.8)	1.000
Active smokers	1,441	34.6 (-11.7~80.9)	0.143	1,450	53.8 (5.5~102.1)	0.023
Smoking duration						
0 year	1,407			1,405		
≤10 years*	1,227	-130.4 (-231.7~-29.0)	0.012	1,435	29.4 (-68.7~127.4)	1.000
>10years*	1,494	86.6 (31.6~141.6)	0.002	1,462	56.4 (4.4~108.7)	0.029

[#]Age, HR, DBP, serum glucose, BMI, TGs, and gender were adjusted for analysis.

*The 39 persons who denied being smokers were excluded.

baPWV, brachial-ankle pulse wave velocity; CI, confidence interval. Other abbreviations see in Table 1.

Table 3. Comparison of baPWV (cm/s) Between Male and Female in Different Age Groups

Smoking status	<50 years			>50 years		
	Male (n=254)	Female (n=225)	P value [#]	Male (n=210)	Female (n=217)	P value [#]
Nons-mokers	1,300 (1,268–1,332)	1,232 (1,208–1,257)	0.001	1,525 (1,478–1,572)	1,583 (1,547–1,620)	0.054
Passive smokers	1,277 (1,230–1,323)	1,225 (1,156–1,293)	0.150	1,457 (1,344–1,571)	1,549 (1,398–1,699)	0.378
Active smokers	1,330 (1,304–1,357)	1,294 (1,213–1,376)	0.408	1,573 (1,517–1,629)	1,562 (1,419–1,705)	0.887

baPWV are mean (95%CI).

[#]Age, HR, DBP, serum glucose, BMI, TGs, and gender were adjusted for analysis.

Abbreviations see in Tables 1, 2.

smokers were 2.91 ng/ml and 147.62 ng/ml, respectively. Among the active smoking people, 179 subjects were male, while 385 females were identified to be non-smokers. The distribution of sexes among various smoking groups is significantly different ($\chi^2=156.03$, $P<0.001$). Of 639 people who reported no cigarette smoking, according to the cotinine level, 542 (85%) were affirmed as non-smokers, 58 (9%) had evidence of being marked passive smokers, and 39 (6%) were reclassified as active smokers. The characteristics of the participants are listed in **Table 1**. Passive smokers were 4.5 years younger on average than non-smokers. Active smokers vs non-smokers showed significantly higher BMI. Compared with passive smokers, active smokers had a higher SBP (132 vs 124 mmHg, $P<0.001$) and DBP (82 vs 78 mmHg, $P=0.002$). Higher levels of TC were found in non-smokers vs passive smokers. Active smokers vs passive smokers had higher TG and glucose levels. Active smokers obviously had a higher morbidity of hypertension than the other subjects.

Correlation of Cotinine and baPWV

In the overall study population, the partial correlation coefficient was 0.097 between baPWV and the serum cotinine level ($P=0.013$), controlling for age, HR, DBP, serum glucose, BMI, TG, and gender. A significant partial correlation coefficient of 0.09 was found in Julu residents ($P=0.024$).

Comparison of baPWV Among Different Smoking Statuses and Different Smoking Durations

As is shown in **Table 2**, active smokers vs non-smokers showed significantly higher baPWV (1,450 vs 1,396 cm/s, $P=0.023$) after adjusting for covariates, and participants who smoked longer than 10 years had a higher baPWV than

non-smokers (1,462 vs 1,405 cm/s, $P=0.029$), although no significant differences existed between those smokers who had a smoking history less than 10 years and non-smokers. No significant differences existed between passive smokers and non-smokers in this study, even after adjusting for covariates.

Comparison of baPWV Between Males and Females

Subjects were divided into 2 groups according to age (**Table 3**). For young non-smokers (<50 years), men tended to have a higher baPWV than women ($P=0.001$), while no significant differences existed between the 2 sexes for active smokers in this age group ($P=0.408$). Male vs female had no significant differences between various smoking states in older groups.

Discrepancy of baPWV Between Julu and Beijing Participants in Various Smoking States

For non-smokers, citizens in Beijing had a higher mean value of baPWV than dwellers in Julu, even after adjusting for covariates (1,495 vs 1,370 cm/s, $P<0.001$). The discrepancy was less in passive and active smokers between Beijing and Julu participants ($P=0.190$ and $P=0.147$).

Comparison of baPWV Among Different Smoking Statuses in Poor Areas

The smoking rate was higher in Julu than in Beijing participants (24.7% vs 12.3%, $P<0.001$). Thirteen percent of non-smokers from Julu were exposed to heavy passive smoke, much higher than the rate (5.26%) in Beijing ($P<0.001$). Further analysis, as demonstrated in **Figure**, shows that active smokers vs non-smokers in Julu had a higher baPWV after adjusting for covariates ($P=0.017$). No significant differences existed among various smoking groups in

Figure. Comparison of the brachial-ankle pulse wave velocity (baPWV) among different smoking statuses of participants from a less developed area in North China. Active smokers had significantly a higher baPWV than non-smokers after adjusting for age, heart rate, diastolic blood pressure, serum glucose, body mass index, triglyceride, and gender ($P=0.017$).

Beijing ($P>0.05$) after adjusting for covariates.

Discussion

Smoking is the leading modifiable risk factor for cardiovascular disease.^{23,24} Many mechanisms are responsible for arterial stiffness caused by cigarette exposure: smoking causes injury to the vascular endothelium, produces superoxide anions, reduces production and bioavailability of nitric oxide, increases production and release of endothelin, causes endothelial dysfunction, and generates pro-inflammatory alterations.^{25–29} In accordance with other research,^{3,4,14} we verified in the present study that active smoke can aggravate arterial stiffness. However, compared with previous results using the questionnaire method, we deduced that smoking status judged by a biochemical indicator can be relatively more objective.^{18–21}

Nicotine is responsible for tobacco addiction and is the most specific component of cigarette smoke. It is absorbed and is measurable in both active and passive smokers. However, nicotine levels in the blood fluctuate and the half-life of nicotine is approximately 2 h and hence it is poorly suited as a marker for monitoring chronic exposure. Cotinine is the major metabolite of nicotine, and is the analyte of choice as it fulfils the prerequisites of specificity and retention time.^{30–32}

According to the serum cotinine level, we found that several active smokers (6%) had a trend of denying smoking during questioning, and some self-reported non-smokers were identified to be heavily exposed to environmental smoke, so the use of cotinine was very helpful in our epidemiological study.

In the present study, cotinine verified that active smokers had significantly higher baPWV values after adjusting for covariates. Furthermore, to determine the relationship between arterial function and smoking duration, we extracted current smokers for further analysis, and those participants where cotinine verified them as being active smokers who initially denied being smokers were excluded from analysis because they lacked to report their smoking habit details.

Participants who smoked for longer than 10 years were found to have worse arterial stiffness, denoting that arterial aging and stiffening might be aggravated by a long history of smoking. In fact, the beginning age of cigarette smoking is very crucial to arterial health.³³ Controlling the smoking rate in younger generations is an imperative task for developing countries. Recommendation of smoke cessation also should be given to new smokers, whose arterial problems are reversible before structural changes have been established.³⁴

On the basis of the present study, arterial elasticity is better for women than men when they are younger than 50 years. This is in accordance with previous research,^{35–37} which suggests that estrogen might have beneficial effects on arterial stiffness.³⁸ However, in the present study, the baPWV of young female smokers tended to be the same as their male counterparts, which implies smoking will minimize the advantage a woman has before menopause. Therefore, we can deduce that smoking can influence the arterial function of young females more seriously than it does for males.

With respect to the comparison between passive smokers and non-smokers, no significant difference in baPWV was found after adjusting for covariates. This is associated with the better health conditions for passive smokers in the present study. For example, passive smokers in the present study have much lower rates of hypertension (27.7%) and diabetes (7.2%) than non-smokers, for whom the rates of hypertension and diabetes are 37.8% and 10.4%, respectively. Meanwhile, although our cut-off value for passive/active smokers (15 ng/ml) has been accepted by many studies,^{15,20,22} there is still no real consensus on a precise target value, with ranges from lower than 10 ng/ml to higher than 25 ng/ml.^{18,39–42} Considering that the median value of cotinine levels for active smokers (147.62 ng/ml) was very high, it is reasonable to presume that some heavy second-hand cigarette smokers in the present study were misclassified as active smokers, which would contribute to concealing the impact of passive smoke to some extent. Accordingly, we cannot conclude that passive smoking has no bad effect on vascular function or doubt the usage of cotinine to diagnose passive smoking. On the contrary, using cotinine to discover more second-hand smokers is very useful in carrying out research for large populations, which could also investigate and confirm the influential effect of passive smoking on arterial health for the general public.

Cigarette smoking is particularly prevalent in mainland China, especially in Chinese males.⁴³ As one of the poorest towns supported by national funds, Julu had been thought to have a lower smoking rate than other developed areas in China. However, according to the present study, the tobacco exposure rate in this poor town was surprisingly higher than that in Beijing. Further analysis demonstrated that non-smokers in Beijing had a higher mean value of baPWV than dwellers in Julu, but the discrepancies were lessened in passive and active smokers between the 2 areas. Furthermore, although active smoking contributed to significant arterial stiffness in Julu participants, a similar phenomenon could not be found in modernized Beijing, even though the exposure degree was almost the same between Beijing and Julu participants in passive smokers and active smokers (median cotinine level 2.76 vs 2.92 ng/ml, $P=0.795$ and 148 vs 144 ng/ml, $P=0.889$). Therefore, we believe that chronic smoke exposure brings more harm to the arterial health for farmers living in poor areas than it does for citizens from

developed cities in North China. A genetic difference is unlikely in the present study, as Beijing is completely geographically surrounded by the Hebei province and the majority of subjects studied in Beijing originated from Hebei. Compared with serial studies conducted with South Chinese participants by Kam et al^{5,29} which indicated that both active and passive smoking had less harmful effects on endothelium-dependent and endothelium-independent arterial dilation for rural people, we seem to have found different results, and this is very likely to be related to the huge contrast between the 2 areas studied. In the present study, Beijing, the host city of the 2008 Olympic Games, experienced a booming economy during these years. Meanwhile, as one of the most prosperous cities in Mainland China, with almost all of the best domestic universities clustered there, Beijing people have a high cultural quality. The condition is totally opposite for the region of Julu. As one of the poorest areas in China, the participants coming for this medical examination are farmers living nearby, whose incomes are the lowest in the country. Consequently, knowledge and treatment rates of chronic disease are very low, and imported medicine useful to relieve arterial stiffness and atherosclerosis including angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers, and statins are not commercially available there. All of these factors make the apparently younger countryside dwellers more vulnerable to the harmful effects of cigarette exposure, presenting as the relatively higher baPWV value for smoke-exposed people in the rural area.

Study Limitations

One shortcoming of using cotinine to determine smoking states is that it can not reflect the accurate condition for a very long period of time, because of its limited existence in the blood, with a half-life of 20 h;⁴⁴ however, it does have a longer existence than nicotine. So in terms of very long-term smoking conditions, we have to believe that people's smoking state remains stable over a long period, which might influence the accuracy of our results.

Conclusions

Active smokers, as confirmed by the use cotinine, have worse arterial stiffness, and long-term smoking (>10 years) can cause marked arterial stiffness. Active smoking might influence the arterial function of young females more seriously than their male counterparts. We should pay close attention to the underestimated smoking rate in less developed areas, as cigarette smoke exposure might bring about more serious effects on arterial stiffness in participants from poor areas than those from modernized cities in North China.

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