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Original article

Effect of nicotine on the release of lactate dehydrogenase and the production of tumor necrosis factor-α of cultured macrophage

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Background: Nicotine can affect the development of Atherosclerosis (AS). Monocytes/macrophages are the important cells in the AS lesions.
Objective: We studied the mechanisms of smoking on AS. The effects of nicotine on macrophage were investigated in this study.
Methods: Different concentration of nicotine ($6 \times 10^{-9\sim-5}$ mol/L), different incubation time (3, 6, 9, 12, 18, and 24 hours) and 7 β-hydroxycholesterol (50 μg/ml) were schemed in this study. After exposure of macrophage to those different conditions, lactate dehydrogenase (LDH) activity and tumor necrosis factor-□ (TNF-α) content in the supernatant were assayed.
Results: Nicotine (6×10^{-9}mol/L~6x10^{-5}mol/L) treatment resulted in a marked reduction of LDH in the supernatant (131.0±9.6 U/L, 129.7±6.2 U/L, 129.4±5.3 U/L, 134.2±8.4 U/L, and 138.3±9.7 U/L vs. 151.3±8.1 U/L, p <0.05 respectively, q-test). The same change trend was seen when co-treated with 7β-hydroxycholestrol and nicotine (135.7±7.6 U/L, 135.6±6.6 U/L, 136.1±6.7 U/L, 142.9±4.5 U/L, and 146.4±4.4 U/L vs. 152.4□6.2 U/L, P<0.05 respectively, q-test). The peak effects occurred at the nicotine concentration of 6×10^{-7}mol/L and the first 18-hours incubation. Nicotine (6×10^{-9}mol/L~6×10^{-6}mol/L) treatment result in the increase of TNF-α in the supernatant (0.28±0.06 ng/mL, 0.32±0.05 ng/mL, 0.40±0.07 ng/mL, and 0.30±0.08 ng/mL vs. 0.17±0.05 ng/mL, p <0.05 respectively, q-test). Nicotine (6×10^{-5}mol/L) treatment have no significant increase compared to the control group (0.21±0.08 ng/mL vs. 0.17±0.05 ng/mL, p >0.05, q-test). The peak effects occurred at the nicotine concentration of 6×10^{-7}mol/L.
Conclusions: Nicotine can produce the beneficial effect on macrophage. Nicotine treatment can activate macrophage to produce TNF-α. Thus, nicotine can be a mechanism on the development of atherosclerosis.

Keywords: Lactate dehydrogenase, macrophage, nicotine, tumor necrosis factor-α

Atherosclerosis (AS) is an important disease worldwide [1, 2]. In the AS lesions, monocytes and monocyte-derived macrophages are the important cell constituents [3-5]. Macrophages have numerous effects that may affect atherosclerosis [4]. The two most important effects are removing the lipid in the lesions and inflammatory reaction.

Smoking is an independent risk factor of AS. Nicotine is the predominant constituent in cigarette smoke. As a toxic substance, studies showed that nicotine could impair the cells [6, 7]. However, some other studies confirmed that nicotine treatment was favorable to the cells' culture [8, 9]. The results of nicotine on macrophage can affect its physiological functions seriously.

Inflammation is the key step in the development of AS. Animal experiment showed that nicotine could augment atherosclerotic lesion and inflammation [10]. Researches also showed nicotine could activate macrophages directly, impair the endothelial function [11], up-regulate the VEGF expression [12], and increase the monocyte adherence and diapedesis [13, 14].

However, as a cholinergic agonist, research found that nicotine could inhibit the inflammation and chronic inflammatory diseases [15, 16]. In the vessel wall, nicotine can block endothelial cell activation and leucocyte recruitment [17], and suppress dendritic cell

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function [18, 19]. Based on these results, nicotine may inhibit the inflammatory in the atherosclerotic lesions, and inhibit the development of AS. Thus, there is contradiction on the effect of nicotine on the inflammatory system in the AS lesion. There are seemingly conflicting and non-reconcilable findings on the mechanisms of nicotine on AS. In this study we want to find out the direct and indirect effects of nicotine on the macrophage, which will illustrate some mechanisms of smoking on the development of AS.

Materials and methods

All experiments conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, revised 1996).

Animals

Adult New Zealand white rabbits weighing 2 to 3 kg were obtained from the experimental animal center of Wuhan University. All animals were maintained on a 12 hours light-dark cycle and were allowed free access to food and water for at least one month before being used in this study.

Laboratory apparatus and methods

RPMI-1640, fetal calf serum, HBSS, and other laboratory reagent were obtained from GIBCO. Nicotine and 7β-hydroxycholesterol were purchased form SIGMA. Cytotoxicity Detection Kit was the production of SUBIO. TNF-α ELISA Kit were from Academy of Military Medical Science of the CPLA. All reagents were of analytical purity grade.

Macrophages collect and culture

Macrophage was derived from blood monocyte. PBM (peripheral blood monocytes) were obtained by centrifugation of blood on Ficoll-Hypaque gradients described as previous data [20]. Cells were plated on 24-well plates at a density of 0.5ml (5×10^5 cells per well). After plating, the cells were grown to confluence. Medium was then replaced and cells treated appropriately.

Experimental design

Concentration related effect of nicotine on macrophage Nicotine was added into the culture medium, and adjusted until the final nicotine concentration was $6\times10^{-9\sim-5}$mol/L, respectively. In the control group, the equivalent to RPMI-1640 was added. The supernatant was aspirated after 24-hour incubation for LDH assay.

Effect of hydroxycholesterol on the macrophage incubated with the nicotine

Nicotine and 7β-hydroxycholesterol (50 ug) were added into the culture medium, and nicotine was adjusted until the final concentration was $6\times10^{-9\sim-5}$mol/L. In the control group, nicotine was added equivalent to RPMI-1640. The supernatant was aspirated after the 24-hour incubation for assaying LDH.

Time-related effect of nicotine on macrophage

Nicotine was added into the culture medium, and the nicotine adjusted until the final concentration was 6×10^{-7}mol/L. In the control group, the equivalent to RPMI-1640 was added. The supernatant was acquired for assaying LDH at 3, 6, 9, 12, 18, and 24-hour after incubation.

Effect of nicotine on the TNF-□ production of macrophage

Nicotine was added into the culture medium and adjusted until the final nicotine concentration was $6\times10^{-9\sim-5}$mol/L. Nicotine was added to the control group equivalent to RPMI-1640. The supernatant was aspirated after the 24-hour incubation for assaying TNF-α.

Measurements made

TNF-α levels in cell-free supernatant were determined by ELISA using commercially available matched antibody pairs following a protocol furnished by the manufacturer. TNF-α concentrations were measured at 450 nm with a reference filter at 550 nm. The results expressed as ng/ml. The cell-free supernatant was collected respectively. The LDH released in the supernatant were determined via the Cytotoxicity Detection Kit following the manufacturers' instructions. The results expressed as U/L.

Data analysis and statistical procedures

All data are presented as means□standard deviation. Statistical analysis was performed using a t-test for unpaired data or one-way analysis of variance (ANOVA) for unpaired data followed by Bonferroni's test, as appropriate. Differences with $p < 0.05$ were considered significant.

Results

Effect of different concentrations of nicotine on LDH release from macrophage

Compared to the control group, the LDH content in the supernatant of the nicotine group were lower than the control group (131.0±9.6U/L, 129.7±6.2U/L, 129.4±5.3U/L, 134.2±8.4U/L, and 138.3±9.7U/L vs. 151.3±8.1 U/L ÿ, p <0.05 respectively, q-test). The results can be seen in **Table 1**.

The LDH content decreased gradually from the nicotine concentration of 6×10^{-9}mol/L to 6×10^{-7}mol/L firstly (131.0±9.6U/L, 129.7±6.2U/L, and 129.4±5.3U/L, p >0.05 respectively, q-test), then increased at the nicotine concentration of 6×10^{-6}mol/L and 6×10^{-5} (134.2±8.4U/L, 138.3±9.7U/L). At the nicotine concentration was 6×10^{-7} mol/L, the LDH content in the supernatant was the lowest among the 6 groups (**Figure 1**).

Effect of hydroxycholesterol on the effect of nicotine

In nicotine+ hydroxycholesterol group, the LDH content of the supernatant were lower in the nicotine group than the control group (135.7±7.6 U/L, 135.6±6.6 U/L, 136.1±6.7 U/L, 142.9±4.5 U/L, and 146.4±4.4 U/L vs. 152.4±6.2 U/L, p <0.05 respectively, q-test).

Table 1. The content of LDH in the supernatant of different concentration of nicotine (X±S) (n = 40).

Concentration (mol/L)	LDH content (U/L)	
	Nicotine group*	Nicotine+ hydroxycholesterol group*
Control group	151.3±8.1	152.4±6.2
6×10^{-5}	138.3±9.7	146.4±4.4ΔΔ
6×10^{-6}	134.2±8.4	142.9±4.5ΔΔ
6×10^{-7}	129.4±5.3	136.1±6.7Δ
6×10^{-8}	129.7±6.2	135.6±6.6Δ
6×10^{-9}	131.0±9.6	135.7±7.6Δ

Values are means±SD. *p >0.05 among 6×10^{-9}~6×10^{-7} mol/L; p <0.05 among the other groups (q-test). Δp <0.05, ΔΔp <0.01 between the nicotine group and the nicotine+hydroxycholesterol group (t-test).

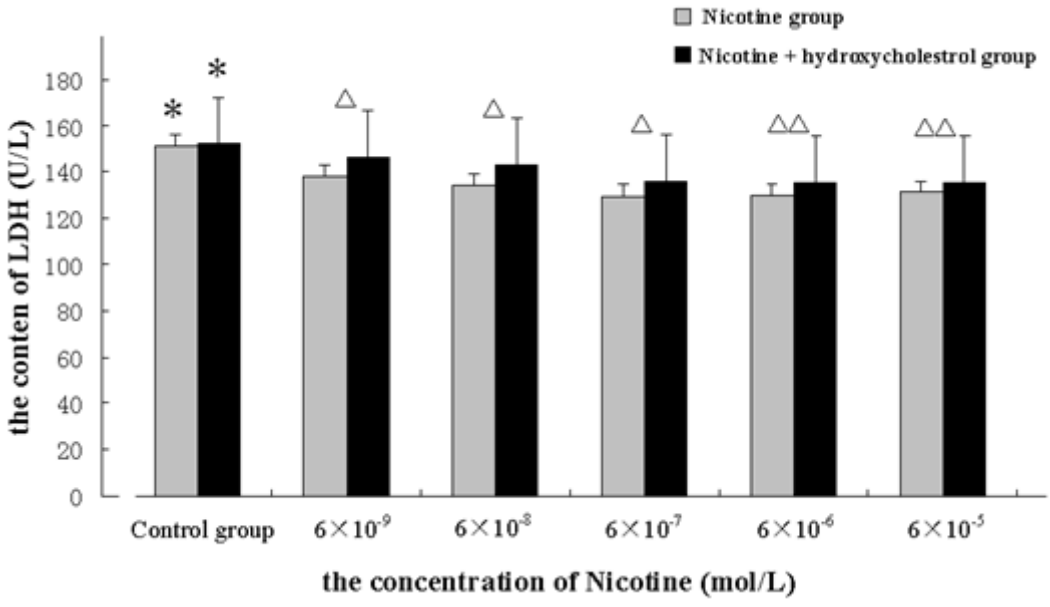

Figure 1. Means±SD the content of LDH in the supernatant of different concentration of nicotine (n = 40). *p <0.05 vs. the control group (q-test). Δp <0.05 ΔΔp <0.01 between the nicotine group and the nicotine+ hydroxycholesterol group at the same nicotine concentration (t-test).

The LDH content was increased with the increase of the nicotine concentration of 6×10^{-9}mol/L to 6×10^{-5}mol/L (135.7±7.6 U/L, 135.6±6.6 U/L, 136.1±6.7 U/L, 142.9±4.5 U/L, and 146.4±4.4 U/L). However, at the nicotine concentration of 6×10^{-9}~6×10^{-7} mol/L, there were no significant difference (135.7±7.6 U/L, 135.6±6.6 U/L, and 136.1±6.7 U/L, P>0.05 respectively, q-test).

When treated with 7□-hydroxycholesterol, there was no significant difference between the two control groups (152.4±6.2 U/L vs. 151.3±8.1 U/L, p >0.05 respectively, t-test). When co-treated with 7β-hydroxycholesterol and nicotine, the LDH content of the supernatant was higher than the group that was nicotine treated (146.4±4.4 vs.138.3±9.7, and 142.9±4.5 vs.134.2±8.4, p <0.01 respectively, t-test; 136.1±6.7 vs.129.4±5.3, 135.6±6.6 vs.129.7±6.2, and 135.7±7.6 vs.131.0±9.6, p <0.01 respectively, t-test) as shown in **Figure 1**.

Effect of different incubation time of nicotine on the LDH released from macrophage

In the nicotine group, the LDH content in the supernatant was lower than the relative time group in every incubation time. However, there were no significant difference of LDH between the control group and the nicotine group in 1, 3, 6, 9-hour incubation (122.4±4.2 U/L vs. 122.7±4.5 U/L, 122.7±2.9 U/L vs. 120.8±4.8 U/L, and 123.2±4.3 U/L vs. 120.0±4.3 U/L, p >0.05, t-test). There were significant differences of LDH at the 12-hour incubation (129.1±5.6 U/L vs. 120.7±4.9 U/L, p >0.05 respectively, t-test) and at the 18, 24-hour incubation (141.5±5.7 U/L vs. 122.8±2.7 U/L, and 152.9±5.8 U/L vs. 130.3±3.4 U/L, p <0.01 respectively, t-test). As shown in **Figure 2**.

In the control group, there were no significant difference among the first 3, 6, 9-hour incubation (122.4±4.2 U/L, 122.7±2.9 U/L, and 123.2±4.3 U/L, p >0.05, q-test), while in the nicotine group, there were no significant differences among the 3, 6, 9, 12, 18-hour incubation (122.7±4.5 U/L, 120.8±4.8 U/L, 120.0±4.3 U/L, and 120.7±4.9 U/L, p >0.05, q-test) (**Table 2**).

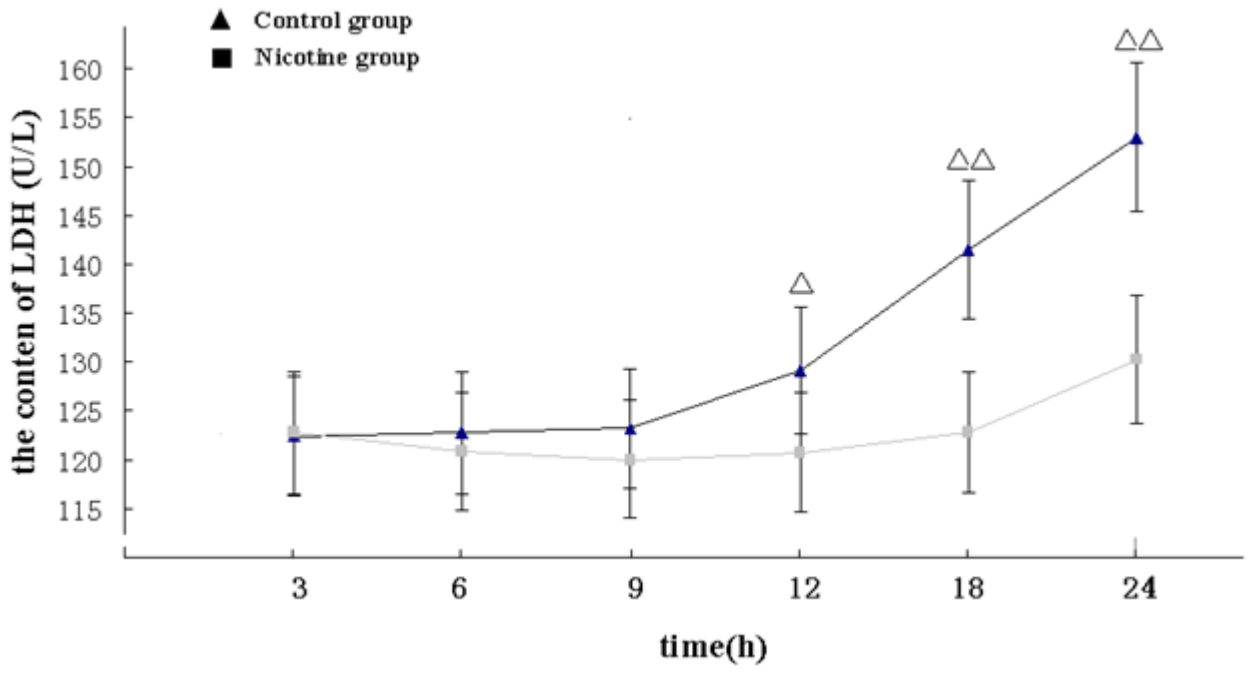

Figure 2. Means±SD the content of LDH in the supernatant of different incubate time of the 6×10^{-7}mol/L nicotine (n = 40). Δ p <0.05, ΔΔ p <0.01 between the nicotine and control group at same incubation time (t-test).

Table 2. The content of LDH in the supernatant of co-incubate time of the 6×10^{-7}mol/L nicotine (n = 40).

Time (hour)	LDH content (U/L)	
	Control group*	Nicotine group#
3	122.4±4.2	122.7±4.5
6	122.7±2.9	120.8±4.8
9	123.2±4.3	120.0±4.3
12	129.1±5.6	120.7±4.9Δ
18	141.5±5.7	122.8±2.7ΔΔ
24	152.9±5.6	130.3±3.4ΔΔ

Values are means±SD. Δ p <0.05, ΔΔ p <0.01 between the same incubation time group (t-test). * p <0.05 among the incubation time of 3, 6, and 9 hours; # among the incubation time of 3, 6, 9, 12, and 18 hours (q-test).

Effect of different concentration of nicotine on TNF-α secreting from macrophage

In the nicotine group the content of TNF-α was more than the control group (0.28±0.06 ng/mL, 0.32±0.05 ng/mL, 0.40±0.07 ng/mL, and 0.30±0.08 ng/mL vs. 0.17±0.05 ng/mL, p <0.05 respectively, q-test). There were no significant differences between 6×10^{-5} group and control group (0.21±0.08 ng/mL vs. 0.17±0.05 ng/mL, p >0.05, q-test) see **Figure 3**.

At the nicotine concentration of 6×10^{-7}mol/L, the TNF-α content in the supernatant (0.40±0.07 ng/mL) was the highest among the 6 groups (**Table 3**).

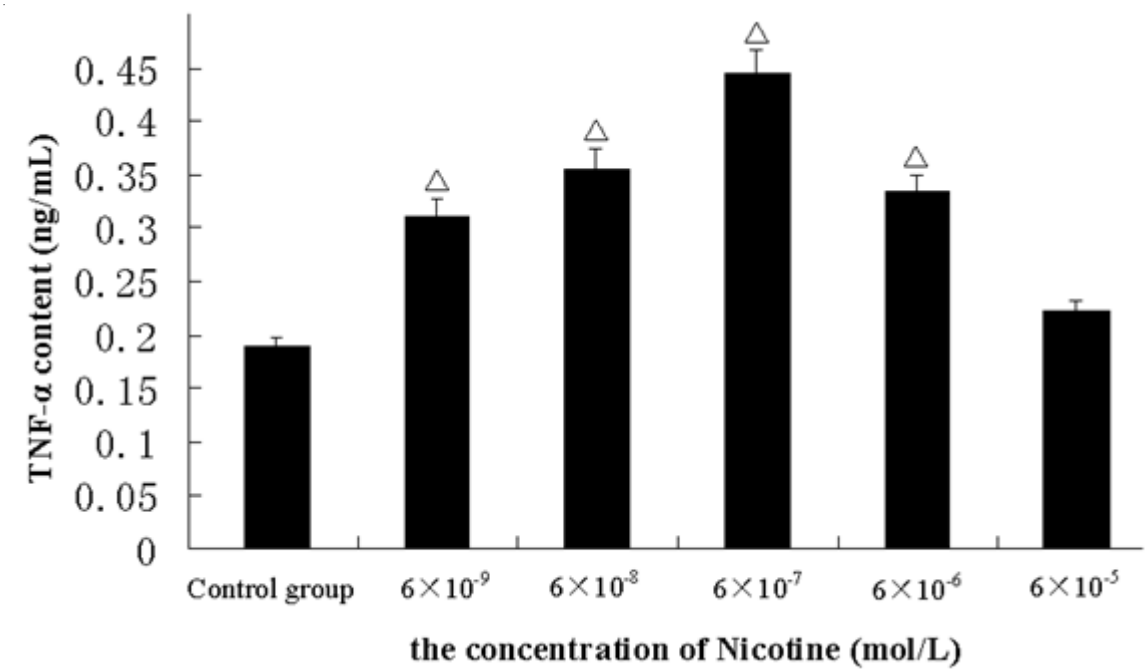

Figure 3. Means±SD the content of TNF-a in the supernatant of different concentration of nicotine (n = 40). Δ p <0.05 vs. control group (q-test).

Table 3. The content of TNF in the supernatant of different concentration of nicotine (n = 40).

Concentration (mol/L)	Control group	6×10^{-9}	6×10^{-8}	6×10^{-7}	6×10^{-6}	6×10^{-5}
TNF-α content (ng/mL)*	0.17±0.05	0.28±0.06	0.32±0.05	0.40±0.07	0.30±0.08	0.21±0.08

Values are means±SD. *p >0.05 between control group and 6□10^{-5}mol/L group, among 6×10^{-9} mol/L, 6×10^{-8} mol/L, and 6×10^{-7} mol/L; p <0.05 among other groups (q-test).

Discussion

Nicotine is an oily liquid that is water-soluble. It can easily enter into the bloodstream and penetrate the tissues. Therefore, nicotine could affect the monocyte in the blood and the monocyte-derived macrophage in the AS lesions. Thus, AS will be affected.

Nicotine is a toxic substance. Previous studies showed that nicotine could impair the cells directly, such as endothelium cells [6, 7]. However, other studies showed that nicotine could prolong the cells survival, such as neutrophil [8] and smooth muscle cells [9]. The most probable reason is that nicotine can combine with the cells through non-cholinergic nicotine receptors. If macrophage was impaired, most of its physiological functions will be affected.

In this study, the LDH content in the supernatant reduced after nicotine treatment. The peak effect occurred at the nicotine concentration of 6×10^{-7}mol/L. This nicotine concentration is the usual blood concentration in most smoking people [21, 22]. In addition, even when the nicotine concentration was up to 6×10^{-5} mol/L, this effect can be found. These observations indicated that nicotine could play a protective effect on the macrophage.

However, when the nicotine concentration was up to 6×10^{-6}mol/L and 6×10^{-5}mol/L, the LDH level of the supernatant was greater than in the other low concentration nicotine group. It showed that the protective effect did not increase with the increasing concentration of nicotine. Thus, this might indicate that the high concentration of nicotine might impair macrophage. Therefore, we can conclude that the effect of nicotine on macrophage is decided by its concentration. When macrophage was exposed to the low concentration of nicotine (below 6×10^{-7} mol/L), the final effect was protection. If this concentration of nicotine (6×10^{-7} mol/L in this study) was exceeded, the final effect will be impaired.

At the identical incubation time, the release of LDH is lower in the nicotine group than in the control group. When treated with nicotine (6×10^{-7}mol/L), there were no significant differences of LDH content among the first 18-hour incubation. While in the control group, there was no significant difference only in first 9-hours. Therefore, the effect of nicotine on the cultured macrophage was time-related.

In the AS lesion, there are many disadvantage to macrophage. Oxidized low-density lipoprotein (OX-LDL) is a main disadvantage. The hydroxycholestrol, oxidized production of the lipid frame and the cholesterol can produce the toxic effect on macrophage. Compared in the control group, hydroxycholesterol (50 g/ml) has little impaired effect on the cultured macrophage. However, when co-treated with hydroxycholestrol, the LDH level in the supernatant was clearly higher than nicotine treatment alone. The observations showed that hydroxycholesterol would influence the effect of nicotine on macrophage greatly.

The mechanisms may be in the specific receptor in the membrane of the macrophage. The impaired factor, such as the OX-LDL, can damage the cells membrane directly. In addition, the receptors in the membrane will be influenced. Therefore, in our study, it can be found that hydroxycholestrol can weaken the effect of nicotine. When macrophage was impaired, the function of these receptors will be initially influenced. Therefore, in the AS lesion, macrophage will lose most of its abilities, particularly the migration. Thus, will make the impaired macrophage stay in the AS lesions, keep on up-taking lipid, and become foam cells.

As immunocyte, macrophage can secrete cellular metabolistic materials and activity components. TNF-α is one of the most important pre-inflammatory media. It can affect the other cells and initiate the inflammatory system [23]. In this study, nicotine treatment results in the increase the production of TNF- by macrophage. The peak effect was at the nicotine concentration of 6×10^{-7}mol/L, which is the nicotine concentration in ordinary blood in most smoking people. The observation indicated that nicotine could activate macrophage to secrete inflammatory media. This was consistent with previous study [23, 24]. However, most of previous study also showed that nicotine could inhibit the inflammatory reaction because it was an agonist of nACHRs [25-27]. These make a contradictory conclusion about the effect of nicotine. The reason might be the treatment method. In this study, nicotine was applied to affect the macrophage directly.

In conclusion, nicotine treatment can benefit the existence of macrophage and activate macrophage to secrete TNF-α. Thus, in the AS lesion, the nicotine can affect the development of AS.

The authors have no conflicts of interest to declare.

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