

# Effects of quercetin on the nicotine-induced oxidative status in male Wistar rats: study on c-reactive protein (CRP) and malondialdehyde (MDA) concentrations

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## ABSTRACT

Nicotine can cause atherosclerosis by activating nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway lead to induce proinflammatory cytokines release as C-reactive protein (CRP) main regulators. The increase of CRP can induce reactive oxygen species (ROS) and increase of malondialdehyde (MDA). Quercetin has been proven to have antiinflammatory and antioxidant effects. This study was conducted to evaluate effect of quercetin on serum CRP and MDA concentrations in rats induced by nicotine. This was a true experimental study with post test only control group design. Thirty six of male Wistar rats were divided into six groups. Group I as normal control received 1 mL/kg BW of NaCl 0.9% solution. Group II as negative control received 2 mg/kg BW of nicotine and Group III as positive control received 2 mg/kg BW of nicotine and atorvastatin at dose of 5 mg/kg BW. Group IV-VI as treatment groups received 2 mg/kg BW of nicotine and quercetin at dose of 25; 50 or 100 mg/kg BW, respectively. Nicotine was given subcutaneously whereas atorvastatin and quercetin were given orally once per day for 28 days, consecutively. Serum CRP and MDA concentrations were measured using Rat hs-CRP ELISA kit and TBARS assay kit, respectively. Data were analyzed using analysis of variance (ANOVA) continued using LSD post-hoc test. The results showed that quercetin reduced serum CRP and MDA concentrations in dose dependent manner. Serum CRP concentration on Group V ( $173.39 \pm 34.85$  ng/mL) and Group VI ( $114.15 \pm 43.62$  ng/mL) were significantly lower than that Group II ( $244.77 \pm 37.95$  ng/mL) ( $p < 0.05$ ). Furthermore, serum MDA concentration on Group IV ( $5.95 \pm 0.11$  mmol/mL), Group V ( $3.93 \pm 0.09$  mmol/mL) and Group VI ( $2.14 \pm 0.09$  mmol/mL) were significantly lower than that Group II ( $7.29 \pm 0.06$  mmol/mL) ( $p < 0.05$ ). In conclusion, quercetin reduces the nicotine-induced oxidative status in rats.

## ABSTRAK

Nikotin dapat menyebabkan atherosclerosis dengan mengaktifkan jalur *nuclear factor- $\kappa$ B* (NF- $\kappa$ B) yang menginduksi pelepasan sitokin proinflamasi sebagai regulator utama C-reactive protein (CRP). Kenaikan CRP dapat menginduksi *reactive oxygen species* (ROS) dan meningkatkan malondialdehid (MDA). Quersetin terbukti memiliki efek antiinflamasi

dan antioksidan. Penelitian ini dilakukan untuk mengkaji efek quersetin terhadap kadar serum CRP dan MDA tikus yang diinduksi nikotin. Penelitian ini merupakan penelitian eksperimental murni menggunakan rancangan *post test only control*. Tiga puluh enam ekor tikus dibagi menjadi enam kelompok. Kelompok I sebagai control normal menerima larutan NaCl 0.9% 1 ml/kg BB. Kelompok II sebagai control negatif menerima nikotik 2 mg/kg BB. Kelompok III sebagai control positif menerima nikotin 2 mg/kg BB dan atorvastatin 5 mg/kg BB. Kelompok IV-VI sebagai kelompok perlakuan menerima nikotin 2 mg/kg BB dan quersetin berturut-turut 25; 50 dan 100 mg/kg BB. Nikotin diberikan secara subkutan sedangkan atorvastatin dan quersetin diberikan secara oral. Kadar CRP dan MDA serum ditetapkan menggunakan Rat hs-CRP ELISA kit dan TBARS assay kit. Data yang diperoleh dianalisis dengan analisis varian (ANOVA) dilanjutkan dengan tes LSD post-hoc. Hasil penelitian menunjukkan quersetin menurunkan kadar CRP dan MDA yang tergantung dosis. Kadar CRP serum Kelompok V ( $173,39 \pm 34,85$  ng/mL) dan Kelompok VI ( $114,15 \pm 43,62$  ng/mL) lebih rendah secara nyata dari Kelompok II ( $244,77 \pm 37,95$  ng/mL) ( $p < 0,05$ ). Selanjutnya, kadar MDA serum MDA pada Kelompok IV ( $5,95 \pm 0,11$  mmol/mL), Kelompok V ( $3,93 \pm 0,09$  mmol/mL) dan Kelompok VI ( $2,14 \pm 0,09$  mmol/mL) lebih rendah secara nyata dari Kelompok II ( $7,29 \pm 0,06$  mmol/mL) ( $p < 0,05$ ). Dapat disimpulkan, quersetin menurunkan status oksidatif tikus yang diinduksi nikotin.

**Keywords:** quercetin – oxidative status – C-reactive protein – malondialdehyde - nicotine

## INTRODUCTION

Cigarette smoking has become an unhealthy life style almost all over the world.<sup>1</sup> Concerning of one million smokers nowadays, there are about 500 thousand smokers who will die faster. The epidemiology studies estimated that 1 from 3 smokers died due to the cardiovascular disease.<sup>2,3</sup> The main substance which has addictive characteristic in cigarette is nicotine.<sup>4,5</sup> Nicotine exposure on smokers is anticipated to play a significant role in various cardiovascular diseases where endothelial dysfunction appears as the initial sign. Endothelial dysfunction represents the early sign of atherosclerosis disease which is known as inflammation disease on vascular wall.<sup>3,6-10</sup>

Nicotine can trigger the inflammation because it has direct effect on neutrophil and macrophage by activating nicotinic acetylcholine receptor (nAChR) on nerves cell and non nerves cell such as monocyte and endothelium. Nicotine binds to activated macrophage's nAChR, furthermore it will

activate nuclear factor- $\kappa$ B (NF- $\kappa$ B). The activation of NF- $\kappa$ B on macrophage causes the proliferation and migration of vascular smooth muscle cells (VSMC), the production of inflammatory mediators e.g. cyclooxygenase 2 (COX-2), prostaglandin E2 (PGE2), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin- $1\beta$  (IL- $1\beta$ ), inducible nitric oxide synthase (iNOS), and also the expression of adhesion molecules. The release of proinflammatory cytokines to inflammatory sites will trigger the C-reactive protein (CRP) synthesis by macrophage.<sup>6,8</sup>

Synthesis of CRP triggers the increase of free radicals in the body which is known as oxidative stress.<sup>6</sup> Oxidative stress can cause endothelial dysfunction by deactivating nitric oxide (NO) directly, thus it will decrease its bioavailability. It can happen due to superoxide anion ( $O_2^-$ ) which is one of the very reactive free radicals type that binds directly to NO and performs peroxynitric.<sup>11</sup> Besides that, oxidative stress causes tetrahydrobiopterin (BH4) oxidation as one of the NO synthetic

cofactor. This oxidation causes endothelial nitric oxide synthase (eNOS) uncoupling and results in the decrease of eNOS expression and NO production.<sup>12</sup> Despite causing eNOS expression decrease, oxidative stress promotes endothelial cell membranes damage through the mechanism of lipid peroxidation formation marked by the increase of malondialdehyde (MDA) concentration in the body.<sup>13-15</sup>

Atherosclerosis disease induced by inflammation can be prevented by giving the antiinflammation and antioxidant treatments. Quercetin is a flavonol which is mostly found in fruits and vegetables such as apple, black tea, and onion.<sup>16,17</sup> Quercetin has antiatherosclerosis and antithrombosis effects because of its ability as antioxidant, anti platelet, and antiinflammation.<sup>18</sup> Quercetin as an antiinflammation works by blocking NF- $\kappa$ B signal, resulting in decreasing the protein inhibitor (I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ ) phosphorylation.<sup>19,20</sup> It can degrade the NF- $\kappa$ B transcription factor and cytokines expression significantly so that it prevents the increase of CRP concentration.<sup>20</sup> In addition, quercetin works as an antioxidant by catching ROS as the main cause of oxidative stress so that it can prevent NO production decrease and the increase of MDA concentration.<sup>21</sup> This study was conducted to evaluate the effects of quercetin on the concentration of CRP and MDA in nicotine-induced male Wistar rats.

## MATERIALS AND METHODS

### Chemicals

Nicotine bitartrate dyhydrate was obtained from Nacalai Tesque Inc., Japan. Nicotine stock solution was prepared at concentration of 2 mg/mL in NaCl 0.9% solution. Atorvastatin and quercetin were obtained from Sigma-Aldrich, Singapore. Atorvastatin and quercetin stock solutions were prepared

at concentration of 100 mg/mL in sodium carboxy methylcellulose (Na-CMC) 0.5% solution. All of the stock solutions were stored in glass bottle at 4 °C for no longer than 30 days.

### Animal and experimental design

This is a true experimental study with post test only control group design. Thirty six male Wistar rats whose weight ranged between 100 and 150g and age of 5 weeks obtained from the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada were used in this study. The rats were housed in individual cages that were well ventilated under 12-hour cycles of light and dark condition at room temperature. They were fed a standard food and provided free access to water *ad libitum*. After acclimatization period of one week, the rats were divided into six groups with 6 rats in each group. Group I as normal control received 1 mL/kg BW of NaCl 0.9% solution. Group II as negative control received 2 mg/kg BW of nicotine and Group III as positive control received 2 mg/kg BW of nicotine and atorvastatin at dose of 5 mg/kg BW. Group IV-VI as treatment groups received 2 mg/kg BW of nicotine and quercetin at dose of 25; 50 or 100 mg/kg BW, respectively. The nicotine dosage was freshly prepared in NaCl 0.9% solution and subcutaneously injected once per day for 28 days consecutively at 11.00 AM. Whereas, the atorvastatin and quercetin dosages were freshly prepared in Na-CMC 0.5% solution and orally administered once per day for 28 days consecutively, 3 hours before nicotine injection (at 08.00 AM).

### Serum CRP and MDA examinations

On day 35, three hours after the last intervention, rats were anesthetized using

ketamine and diazepam subcutaneously and then were fixated. Blood samples were collected from the rats and placed to the freezer for over 2 hours at room temperature or overnight at 4 °C. The blood samples were then homogenised and centrifuged for 20 min at 3000 g. Serum samples were then collected and stored at -80 °C until analysis. Serum CRP concentration was measured by using Rat hs-CRP ELISA kit, whereas serum MDA concentration was measured by using 2-thiobarbituric acid reactive substances (TBARS) assay kit.

**Data analysis**

Data of serum CRP and MDA concentrations were expressed as mean ± standard deviation (SD) and analyzed by using analysis of variance (ANOVA) continued by least significant difference (LSD) post-hoc test. A p value less than 0.05 was considered significant.

**RESULTS**

Serum CRP and MDA concentrations of all groups are presented in TABLE 1. After

nicotine induction at dose of 2 mg/kg BW for 28 days (Group II), serum CRP ( $244.77 \pm 37.95$  ng/mL) and MDA ( $7.29 \pm 0.06$  mmol/mL) concentrations of the rats increased significantly compared to those without nicotine induction on Group I (serum CRP concentration was  $73.78 \pm 20.94$  ng/mL and serum MDA concentrations was  $0.94 \pm 0.03$  mmol/mL) ( $p < 0.05$ ). After nicotine induction at dose of 2 mg/kg BW and quercetin administrations at dose of 25; 50 and 100 mg/kg BW for 28 days (Group IV-VI), serum CRP concentration on Group V ( $173.39 \pm 34.85$  ng/mL) and Group VI ( $114.15 \pm 43.62$  ng/mL) significantly reduced compared to Group II ( $p < 0.05$ ). Moreover serum MDA concentration on Group IV ( $5.95 \pm 0.11$  mmol/mL), Group V ( $3.93 \pm 0.09$  mmol/mL) and Group VI ( $2.14 \pm 0.09$  mmol/mL) also significantly reduced compared to that Group II ( $p < 0.05$ ). After nicotine induction at dose of 2 mg/kg BW and atorvastatin administration at dose of 5 mg/kg BW (Group III), serum CRP ( $166.65 \pm 19.06$  ng/mL) and MDA ( $3.02 \pm 0.10$  mmol/mL) concentrations also significantly reduced compared to that Group II ( $p < 0.05$ ).

TABLE 1. Serum CRP and MDA concentration in each group

Group	n	Serum CRP (ng/mL)	Serum MDA (nmol/mL)
I (normal)	6	$73.78 \pm 20.94$	$0.94 \pm 0.03$
II (nicotine)	6	$244.77 \pm 37.95$	$7.29 \pm 0.06$
III (nicotine + atorvastatin 5 mg/kgBW)	6	$166.65 \pm 19.06^{a,b}$	$3.02 \pm 0.10^{a,b}$
IV (nicotine + quercetin 25 mg/kgBW)	6	$241.87 \pm 40.30^b$	$5.95 \pm 0.11^{a,b}$
V (nicotine + quercetin 50 mg/kgBW)	6	$173.39 \pm 34.85^{a,b}$	$3.93 \pm 0.09^{a,b}$
VI (nicotine + quercetin 100 mg/kgBW)	6	$114.15 \pm 43.62^{a,b}$	$2.14 \pm 0.09^{a,b}$

<sup>a</sup> significantly lower than Group II ( $p < 0.05$ ); <sup>b</sup> significantly higher than Group I ( $p < 0.05$ ).

Correlation between quercetin doses and serum CRP and MDA concentrations on rats after nicotine induction was also evaluated by using Pearson test of correlation. Negative significantly correlation was observed between quercetin doses and serum CRP and MDA concentrations (TABLE 2).

TABLE 2. Correlation between quercetin doses and serum CRP and MDA concentrations on rats after nicotine induction

Correlation	r	p
Quercetin doses vs CRP concentration	-0.506	0.032
Quercetin doses vs MDA concentration	-0.622	0.006

## DISCUSSION

Endothelial cells have anticoagulant and antithrombotic activities. They also regulate various mediators including NO which is known as endothelium derived relaxing factor (EDRF). NO is synthesized from L-arginine by endothelial nitric oxide synthase (eNOS) in endothelium. NO plays an important role in regulating wide spectrum of cardiovascular functions including mediating vasorelaxation, inhibiting leukocytes-endothelial adhesion and preventing platelet aggregation.<sup>7,21,23</sup> Decrease in NO production caused by endothelial dysfunction can lead to an early sign of various cardiovascular diseases such as atherosclerosis.<sup>6,11</sup>

Inflammation has an important role in the process of atherosclerosis plaque growth until thrombosis occurs that is caused by rupture.<sup>9,25,26</sup> Macrophage located in the plaque contributes to the atherosclerosis occurrence by activating NF- $\kappa$ B to stimulate proinflammatory genes production.<sup>5</sup> Release of proinflammatory cytokines such as IL-6, IL-1, and TNF- $\alpha$  shows the main regulators

from CRP, thus CRP concentration in the body stand for the indicator of a non specific systemic inflammation.<sup>9</sup>

Nicotine can trigger the release of CRP through nAChR in the macrophage which located at atherosclerosis plaque. Synthesized CRP triggers the increase of free radicals which is known as oxidative stress.<sup>6</sup> Oxidative stress can degrade the NO production by deactivating it so that it decreases the NO bioavailability.<sup>11</sup> In addition, oxidative stress causes oxidation of BH4 as one of the NO synthetic cofactor. It causes uncoupling of eNOS and results in decreasing the expression of eNOS and NO production.<sup>12</sup> Oxidative stress also triggers formation of lipid peroxidation which damages endothelial cell membranes, as marked by the increase of MDA concentration in the body.<sup>13,14</sup>

The result indicated that the nicotine administration increased the serum CRP concentration as showed the significantly higher serum CRP concentration after nicotine induction (Group II) compared to without nicotine induction (Group I) ( $p < 0.05$ ). This result is in accordance with previous study which showed that nicotine induction at dose of 2 mg/kg BW cause endothelial dysfunction as indicated by the increase of serum CRP concentration.<sup>5,6,26,27</sup> Nicotine stimulates the production of the inflammatory mediators such as IL-6, IL-1 and TNF- $\alpha$  as main regulator of CRP. Moreover, nicotine induces of CRP production of macrophage by activating the transcription factor NF- $\kappa$ B.<sup>6</sup>

Furthermore, this result also indicated that quercetin administration could inhibit the increase serum CRP concentration induced by nicotine in dose dependent manner (Table 1). Although, the serum CRP concentration after quercetin administration at highest dose (100 mg/kg BW on Group VI) was higher than that without nicotine induction

on Group I. However, this effect of quercetin was significantly higher than atorvastatin as positive control at dose of 5 mg/kg BW (Group III). It was indicated that the quercetin administration at dose 100 mg/kg BW had not prevented inflammation due to nicotine induction, yet. Previous study reported that quercetin reduced the expression of human CRP and cardiovascular risk factors in mice *in vivo*.<sup>19</sup> It was also reported that quercetin reduced serum level of both TNF- $\alpha$  and CRP in models of diabetes.<sup>20</sup>

The effect of quercetin on serum CRP concentration after nicotine induction was parallel with its effect on serum MDA concentration. Quercetin administration could also inhibit the increase serum MDA concentration induced by nicotine in dose dependent manner (Table 1 and 2). Previous studies reported that nicotine induction at the dose of 2 mg/kg BW could stimulate inflammation and oxidative stress lead to an endothelial dysfunction resulting the increase serum MDA concentration.<sup>6,7,26,27</sup> Furthermore, it was reported that quercetin has antiinflammatory and antioxidant effects which may inhibit the inflammation and oxidative stress induced by nicotine.<sup>19,20,21</sup>

## CONCLUSION

It can be concluded that quercetin reduces the oxidative status in rats induced by nicotine as indicated by the decrease of serum CRP and MDA concentrations after quercetin administration. Further study will be conducted to evaluate the mechanism of actions of quercetin in the reduce of oxidative status.

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