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In vivo treatment of acute *Chlamydia pneumoniae* infection with the flavonoids quercetin and luteolin and an alkyl gallate, octyl gallate, in a mouse model

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Abstract

Increasing evidence suggests that plant polyphenolic compounds may protect from cardiovascular diseases, which have been addressed to their antioxidative properties. In addition, these compounds have been shown to possess anti-inflammatory and anti-microbial potential. In the present study we tested the effects of two flavonoid compounds, quercetin and luteolin, and one alkyl gallate, octyl gallate, on the course of acute *Chlamydia pneumoniae* infection in vivo. C57BL/6J mice were treated with quercetin, luteolin or octyl gallate for 3 days prior to and 10 days after *C. pneumoniae* inoculation. Lung tissue was analysed for the presence of chlamydia by culture and quantitative PCR, and inflammatory responses were assessed. Luteolin was found histologically to suppress inflammation in lung tissue, the development of *C. pneumoniae*-specific antibodies and the presence of chlamydia in lung tissue. Octyl gallate had no significant effect on the course of infection, but quercetin increased both the inflammatory responses and the chlamydial load in the lungs. The infection and inflammation-enhancing effects of quercetin treatment may be attributable to the dose and the route of administration and should be reassessed in further studies with lower doses or with different metabolites of the compound. Contrariwise, the effects of luteolin treatment suggest this compound to have potential in decreasing the infection load and inflammatory reactions in vivo.

Keywords: *Chlamydia pneumoniae*; Lung infection; Mouse; Quercetin; Luteolin; Octyl gallate

1. Introduction

Plant polyphenolic compounds, such as flavonoids, are important metabolites of plants in defence against radiation and pathogenic micro-organisms and are present in almost all foods of plant origin, especially teas, onion, apple, berries and red wine [1,2]. Increasing evidence suggests that these compounds may protect from cardiovascular diseases [3,4] and cancer [5]. Numerous studies have so far investigated the possible mechanisms of the health-promoting action of dietary flavonoids. In spite of the suggested effects, including antioxidant properties [6,7], modulation of drug-metabolising enzymes such as cytochrome P450 [8], effects on platelet aggregation [9] and the immune system and antiviral and antibacterial activities [10,11], the essential biological functions of flavonoids are not fully understood yet. One important strategy in flavonoid research is to elucidate the pharmacokinetics and actions of individual compounds and thus to identify those with favourable properties.

Octyl gallate belongs to a group of alkyl gallates, which are synthetic derivatives of gallic acid and are commonly used as antioxidant additives in various foodstuff [7]. Similarly to several flavonoid compounds, alkyl gallates also have antibacterial properties [12]. Quercetin, the most

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widely studied flavonoid in the group of flavonols, and luteolin from the group of flavones are both ubiquitous compounds and microbicidal, as shown in several studies [10,13,14]. All the three compounds studied here have been shown to inhibit phosphorylation cascades and proinflammatory cytokine and chemokine production either in vitro or in animal models [15–18]. In response to an inflammatory stimulus induced by a Gram-negative bacterial endotoxin, lipopolysaccharide (LPS), several phenolic compounds, especially quercetin, attenuate the activation or expression of inducible nitric oxide synthase (iNOS) in macrophages [19]. In addition, quercetin has been shown to significantly lower the high NO levels associated with inflammation in vivo in mice [19].

The intracellular, Gram-negative pathogen *Chlamydia pneumoniae*, which generally causes mild upper respiratory tract infections and occasionally bronchitis and pneumonia, has been suggested to participate in the development of several chronic diseases, such as cardiovascular diseases [20], chronic obstructive pulmonary disease and asthma [21]. *C. pneumoniae* can be found circulating in human peripheral blood mononuclear cells [22] and is known to spread via alveolar macrophages from the lungs to other tissues in mice [23]. Generally in a murine model, intranasal *C. pneumoniae* inoculation leads to acute, self-restricted pneumonia, which is cleared in 3–6 weeks, depending on the animal strain and the inoculum dose [24,25]. Repeated inoculations are associated with aortic changes in normocholesterolemic mice [26], and in hypercholesterolemic mice, inoculations lead to the development of advanced atherosclerotic lesions [27], thus providing further evidence for the association with atherosclerosis. Zhu et al. [28] have proposed the concept of pathogen burden as a risk factor for atherosclerosis in humans, suggesting that the inflammatory responses activated after viral or bacterial infections might contribute to atherogenesis. These responses might be affected by compounds suppressing the bacterial load and inflammation after acute infection.

Our preliminary in vitro studies showed the two flavonoids, quercetin and luteolin, and octyl gallate to be effective in eradicating *C. pneumoniae* in HL cell cultures [29]. The aim of this work was to study whether treatment of mice with these common phenolic compounds modifies the course of acute *C. pneumoniae* infection in vivo.

2. Methods

2.1. C. pneumoniae strain and inoculum

Mice were inoculated with *C. pneumoniae* isolate Kajaani 7 (K7) free of mycoplasma and diluted in sucrose phosphate glutamic acid (SPG) buffer. The inoculum dose was estimated by culturing serial dilutions of the stock in duplicate in HL cells. The cell cultures were done as described earlier [30].

2.2. Animal model

Inbred C57BL/6J female mice purchased from Harlan Netherlands at the age of 6 weeks were divided into five groups of eight mice, and treatments were started at the age of 8 weeks. The treated groups were given 20 mg/kg of quercetin (group 1) (Quercetin dihydrate, Carl Roth, Karlsruhe, Germany), 20 mg/kg of octyl gallate (group 2) (Fluka Chemie, Buchs, Switzerland) and 2 mg/kg of luteolin (group 3) (Extrasynthese, Genay, France). Group 4 was the infected control group, in which the mice received placebo treatment (1% DMSO). The treatments were given intraperitoneally once daily for 3 days prior to inoculation and continued for 10 days post-infection (p.i.). On the fourth day after starting the treatments, the mice in the groups 1–4 were inoculated intranasally with *C. pneumoniae* isolate K7 (7×10^5 IFUs/mouse) under inhaled methoxyflurane (Medical Developments Australia) anaesthesia. The fifth group was inoculated with SPG instead of chlamydia (uninfected controls). Samples were taken on the days 3, 6, 10, 13 and 20 p.i. Another set of experiments were done separately but with a similar study protocol, where only quercetin and luteolin were used to treat the mice, and the samples were collected 4 days p.i. Lung tissue specimens from these mice were collected for the analysis of NOS mRNA expression. The Animal Care and Use Committee of National Public Health Institute, Helsinki, Finland approved all procedures involving animals.

2.3. C. pneumoniae antibody detection

At sacrifice, blood was immediately collected by heart puncture, and serum was separated by centrifugation after the sample had been left to stand at room temperature for 30 min. *C. pneumoniae* total immunoglobulin G (IgG) antibodies were measured with the microimmunofluorescence (MIF) test, using purified, formalin-fixed whole EBs of K7 as antigen. IgG antibodies were detected using Fluorescein Isothiocyanate-conjugated anti-mouse IgG (Serotec).

2.4. Culture of lung tissue

The right lung was kept on ice and mechanically homogenised with 2 ml of SPG for the culture of *C. pneumoniae*. The culture was done as described in more detail previously [31]. The Pathfinder® (Sanofi Diagnostics Pasteur, France) *Chlamydia* genus-specific monoclonal antibody conjugated to fluorescein isothiocyanate was used to detect the chlamydia inclusions in HL cells.

2.5. Detection of chlamydial DNA

The remaining tissue debris separated after the centrifugation of the lung tissue homogenate was stored for chlamydial DNA detection. The reagents for quantitative

DNA and RNA analyses were from Roche Applied Sciences, unless reported otherwise. For the detection of *C. pneumoniae* DNA in lungs, 50 mg of lung tissue debris was lysed with proteinase K in tissue lysis buffer after homogenisation of the tissue with Lysing Matrix D tubes (Q-BIOgene) using the FP 120 FastPrep Cell Disruptor (Savant Instruments, Inc.). After proteinase incubation at 56 °C overnight, DNA was purified using a commercially available QIAamp tissue kit according to the manufacturer's instructions. The purified DNA was kept frozen at −20 °C until analysed. Quantitative analysis of chlamydial DNA was done with the Roche LightCycler system using a FastStart DNA Master Hybridisation Probes kit. Primers for 16S rDNA previously published by Gaydos et al. [32] and specific hybridisation probes for the sequence published by Reischl et al. [33] were obtained from TIB Molbiol (Germany). The sequences for the primers and probes are shown in Table 1. LightCycler PCR reaction mixtures and amplification protocols were as described by Reischl et al. [33], except that 8 μl of template was used in a final volume of 20 μl of reaction mixture. For quantification, *C. pneumoniae* elementary bodies cultured in HL cells were purified by Urografin density gradient ultracentrifugation, and DNA was extracted and analysed using a spectrophotometer. Finally, the numbers of *C. pneumoniae* genomes were calculated using the known molecular weight of one genome. The stock was diluted to obtain standards from 1 to 10^4 genomes/μl, and by using these as a standard curve, the quantification of the samples was calculated by the second derivative maximum method of the LightCycler Data Analysis software (Version 3.5.28).

2.6. Histopathology of the lungs

The left lung was removed and fixed in 10% buffered formalin for histopathological analysis. Formalin-fixed lung specimens were embedded in paraffin, and 4 μm sections were cut and stained with haematoxylin and eosin (HE). The inflammation visible in HE staining was evaluated for the severity of bronchointerstitial pneumonia on a scale from 0 to 4 as described earlier [30].

2.7. Serum nitrate and nitrite analysis

Quantitative determinations of serum nitrate and nitrite were done using the nitric oxide (NO_2^-/NO_3^-) Assay kit (R&D Systems, Minneapolis, MN, USA) in accordance with the kit instructions. Samples were diluted 1:2 and filtered through Microcon® YM-10 Centrifugal Filter Units (Millipore, Bedford, MA, USA) before analysis.

2.8. Quantitative mouse iNOS and eNOS mRNA detection

A piece of about 10–25 mg of the right lung was stored in RNA*later* RNA Stabilization Reagent (Qiagen) in the second set of experiments. The tissue specimen stored in RNA stabilization buffer was weighed, and total RNA was extracted with the HighPure RNA Tissue Kit in accordance with the kit instructions, except that the homogenized tissue was lysed with proteinase K for 1 h at 56 °C before column extraction. DNase was included in the kit protocol to degrade DNA in the samples. Reverse transcriptase (RT) reaction was performed with the Transcriptor Reverse Transcriptase enzyme for 30 min at 55 °C using random $pd(N)_6$ primers according to the manufacturer's recommendations. A quantitative LightCycler analysis for cDNA was performed by the FastStart DNA Master SYBR Green I kit with specific primers obtained from the iNOS [34] and eNOS [35] mRNA sequences (Table 1). The PCR reaction mixtures with a final volume of 20 μl consisted of 2.5 mmol/l $MgCl_2$, 0.5 mmol/l of each primer, 2 μl of FastStart DNA Master CYBR Green I mix, and 2 μl of cDNA template. After pre-incubation at 95 °C, the amplification cycles for the different genes were: iNOS 95 °C/15 s, 61 °C/10 s, 72 °C/10 s and eNOS 95 °C/15 s, 61 °C/10 s, 72 °C/15 s. Specific standards for both genes were produced by purification of the correct PCR product from agarose gel, amplification of this product with LightCycler, and concentration of the amplified products by ethanol precipitation. The amount of specific sequence in each stock was determined and diluted to

Table 1
Primers and hybridisation probes used in DNA and mRNA analyses

Gene (accession no.)	Sequence	Ref.
C. pneumoniae 16S rRNA	CpnA primer: 5′-TGACAACTGTAGAAATACAGC-3′	[32]
	CpnB primer: 5′-CGCCTCTCTCCTATAAAT-3′	
	CP16FL probe: GTAGCAAGATCGTGAGATGGAGCAA-(FL)	[33]
	CP16LC probe: (Red 640)-TCCTAAAAGCTAGCCCCAGTTC-(Ph)	
iNOS mRNA (NM010927)	Forward: 5′-CAGCTGGGCTGTACAAACCT-3′	[34]
	Reverse: 5′-CATTGGAAGTGAAGCGTTTCG-3′	
eNOS mRNA (U53142)	Forward: 5′-TTCCGGCTGCCACCTGATCCTAA-3′	[35]
	Reverse: 5′-AACATATGTCCTTGCTCAAGGCA-3′	
GAPDH mRNA (M32599)	Forward: 5′-AACGACCCCTTCATTGAC-3′	[36]
	Reverse: 5′-TCCACGACATACTCAGCAC-3′	

obtain standards of 1 to 10^4 genomes/μl. Quantification was done by using a standard curve as described in *C. pneumoniae* 16S rDNA LightCycler analysis. A melting curve analysis for each LightCycler run, including the standards, was performed to confirm the amplification of a specific product. In addition, the expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured from the cDNA samples according to the method described by Simpson et al. [36]. The crossing point (Cp) value for the GAPDH and NOS analyses represents the PCR cycle at which the increase in the SYBR Green fluorescence signal above the baseline was detected and indicates the level of template concentration in the sample. The Cp values for GAPDH cDNA in the different study groups (±S.D.) were as follows: uninfected control 24.73 ± 0.99, infected control 25.38 ± 0.99, quercetin 25.06 ± 0.28 and luteolin 26.34 ± 0.48. The GAPDH mRNA levels were affected by infection to some extent, but especially luteolin treatment decreased GAPDH gene expression, which can be seen as an increase in the Cp value. GAPDH was, therefore, not suitable to be used in the relative quantification of NOS. However, a significant negative correlation ($r = -0.524$, $p = 0.006$) was detected between the Cp values for GAPDH and the weight of the tissue taken for RNA extraction, suggesting that tissue weights can be used to adjust mRNA expression levels. We, therefore, adjusted the NOS cDNA absolute quantification calculated by the LightCycler software for tissue weight. The results are presented as per 25 mg of lung tissue.

2.9. Statistical analyses

Spearman's correlation was used to evaluate the correlation between the GAPDH crossing point values from the LightCycler analysis and the weights of the lung tissue specimens taken for analysis. The differences between the groups after *C. pneumoniae* culture and DNA analyses as well as the quantitative NOS expression analyses and plasma nitrate levels were tested with non-parametric Mann–Whitney *U*-test. The lung histopathology scores between the groups were compared with the Chi-square test for trend. Statistics were done using SPSS Version 11.5.1.

3. Results

3.1. Measurement of antibodies

IgG antibodies against *C. pneumoniae* started to appear around day 10 p.i. in the quercetin- and octyl gallate-treated groups. At 20 days p.i., the antibody levels in the luteolin-treated group were significantly lower compared to the untreated controls, and very high levels up to titre 512 were seen in the quercetin-treated mice (Fig. 1).

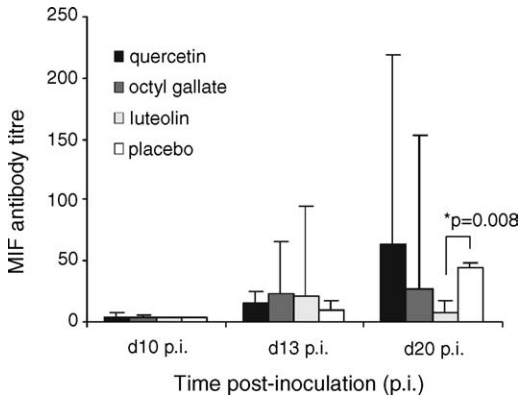

Fig. 1. Geometric means (+95% CI) of the *C. pneumoniae*-specific IgG antibody responses in the different study groups measured by the micro-immunofluorescence method.

3.2. C. pneumoniae culture and DNA detection by PCR from lung tissue

The geometric means of the numbers of viable chlamydia in the culture and the numbers of chlamydial genomes detected by quantitative PCR in lung tissue are shown in Fig. 2A and B, respectively. Each group consisted of eight mice, and variances within the groups were high in both analyses at all time points. For the sake of clarity, variances were not included in Fig. 2A and B. Luteolin showed an attenuating effect especially on the presence of culturable chlamydia in lung tissue compared to the infected control group, and a statistically significant difference was detected on day 10 p.i. ($p = 0.035$, Fig. 2A). Overall positivity, including both culture and PCR-positive findings, was also decreased by luteolin treatments. For example, percentages of the mice found chlamydia-positive in the luteolin-treated groups were 75 and 38% on days 6 and 13, respectively, whereas the corresponding percentages in the infected control group were 100 and 88%. Contrariwise, quercetin treatment increased the presence of both viable chlamydia and chlamydial genomes in lung tissue: significant differences compared to the infected control group were detected on day 6 p.i. in culture analysis ($p = 0.016$, Fig. 2A) and on day 20 p.i. in DNA detection ($p = 0.019$, Fig. 2B). Octyl gallate decreased the numbers of culturable chlamydia in lung tissue on day 3 p.i. ($p = 0.039$, Fig. 2A), but elevated levels were detected on day 6 p.i. No significant differences were found at the other time points. In the second set of experiments with quercetin and luteolin, where the samples were collected 4 days p.i., a significant decrease was detected in the numbers of infectious chlamydia in both the quercetin and luteolin groups (geom. means: 10721, $p = 0.021$ and 8471, $p = 0.020$, respectively) compared to the infected controls (38863 IFUs per 2 ml of lung tissue homogenate). Neither infectious chlamydia nor chlamydial DNA

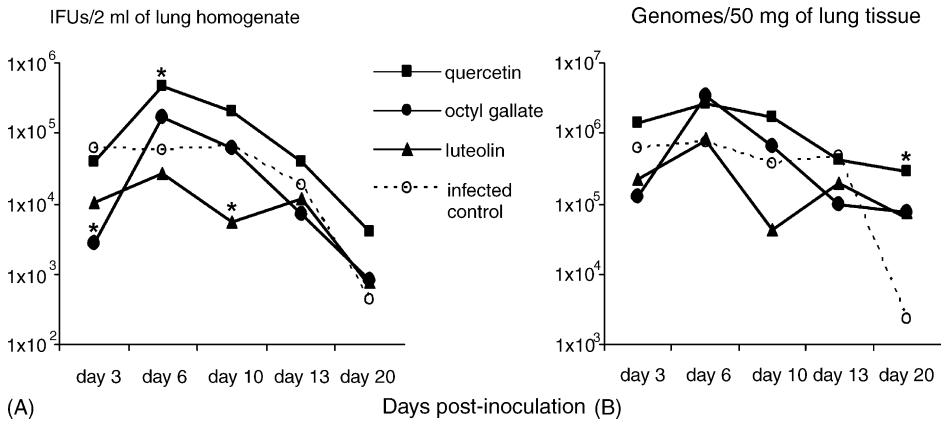

Fig. 2. Quantitative detection of (A) viable chlamydia in lung tissue by culture and (B) the number of chlamydial genomes detected per 50 mg of lung tissue. Dots at lines represent geometric means per study groups. Asterisks show the statistically significant differences of the indicated groups compared to the infected and untreated control groups at the given time point.

was detected in the uninfected control groups in these studies.

3.3. Lung histopathology

The inflammatory reaction in lung tissue consisted of both mononuclear and polymorphonuclear cells on day 3 p.i., but mainly mononuclear cells were detected at the later time points. The results are shown in Fig. 3: 3A shows the percentages of lung inflammation grades in mice in the different study groups on the days 6 and 13 p.i. Fig. 3B presents the average inflammatory scores in the different study groups at all time points. In the first study, luteolin clearly suppressed the lung inflammation detected on the days from 3 to 13 p.i., and the differences were statistically significant on day 13 compared to the infected control group ($p = 0.011$) and the quercetin-treated group ($p = 0.003$). The difference compared to the quercetin-treated group was nearly significant on day 10 as well ($p = 0.075$). A slight attenuation of the inflammation caused by octyl gallate compared to the infected control group was found on the days 3 and 13 p.i. The inflammations in the quercetin-treated mice were similar or worse compared to the infected control mice at all time points (Fig. 3A and B). In the second experiments, luteolin completely abolished the lung inflammation at 4 days

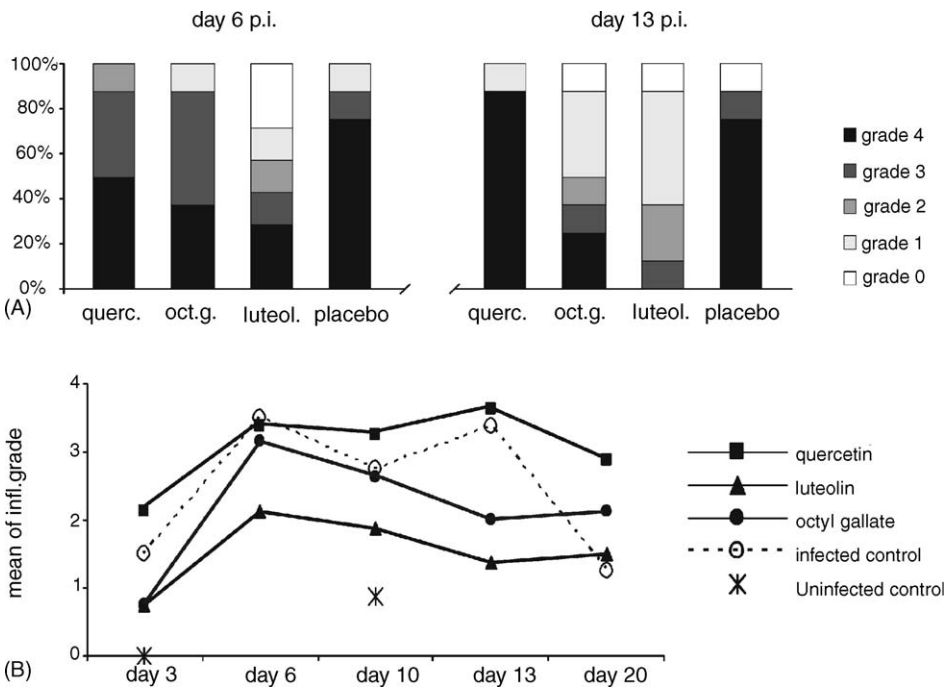

Fig. 3. (A) Percentages of mice with different grades of inflammatory changes in lung histopathology in the study groups on the days 6 and 13 post-infection. *Inflammation significantly milder compared to the quercetin-treated ($p = 0.003$) and untreated control ($p = 0.011$) groups. (B) Averages of inflammatory grades per study group at different time points. Grades: 0, no inflammation; 1, mild changes with lymphocyte and plasma cell infiltration; 2, moderate; 3, marked; 4, severe changes.

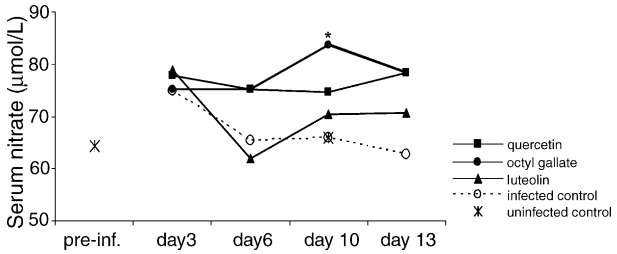

Fig. 4. Geometric means of serum nitrate levels in the study groups. 95% CI of means are presented for the infected control group. Variances were similar in the other groups as well, but were not included in the figure. *Significantly higher compared to the infected control group ($p = 0.037$).

p.i., even though chlamydiae were detected in the lungs of these mice by both PCR and culture (results not shown). No differences were detected between the quercetin-treated and the infected control mice in this study, either.

3.4. Serum nitrate concentrations

The geometric means of the nitrate levels in the study groups at the different time points are shown in Fig. 4. Only nitrate was detectable after the conversion of nitrates to nitrites by the Nitrate Reductase enzyme followed by Griess reaction in the serum samples, whereas the levels of endogenous nitrite were undetectable when the reductase enzyme was not used. Infection itself seemed to increase NO on the days 3 and 6 p.i., and a trend towards an increase in the nitrate levels compared to the infected control group was detected in the quercetin and octyl gallate-treated mice. Yet, statistical significance was found only in the octyl gallate treatment group compared to the infected control group on day 10 p.i. ($p = 0.037$).

3.5. Quantitative expression of iNOS and eNOS mRNA

Absolute quantifications of iNOS and eNOS mRNA per 25 mg of lung tissue on day 4 p.i. from the second set of experiments are shown in Fig. 5. Luteolin treatment significantly decreased the expression of constitutive eNOS enzyme ($p = 0.042$; Fig. 5B) compared to the infected control group, whereas no differences in the iNOS levels between the luteolin and the infected control group were seen (Fig. 5A). Quercetin had no effect on eNOS, but opposite to the general suppressing effect of luteolin, quercetin significantly increased iNOS expression compared to the infected control ($p = 0.028$) and luteolin ($p = 0.019$) groups (Fig. 5A).

4. Discussion

Two common dietary flavonoids, quercetin and luteolin, and octyl gallate from the group of alkyl gallates were administered to *C. pneumoniae*-infected mice, to see whether these compounds could affect the course of acute infection in vivo. Luteolin was found to be effective in suppressing the lung inflammatory response and decreasing the presence of infectious chlamydia in lung tissue. The treatment also lowered the levels of *C. pneumoniae*-specific antibodies in serum. Chlamydia genome numbers were not significantly affected by the treatments in PCR-positive mice, but the presence of chlamydia in lung tissue, i.e. the number of mice found positive by either culture or PCR, was lowest at all time points in the luteolin-treated mice. In the second set of analyses performed in another study, luteolin treatment decreased the expression of the constitutive form of NOS, eNOS, compared to an infected control group. The decreasing effect on the presence of infectious chlamydia and the suppression of lung inflammation after luteolin treatment were also seen in this second study. These are preliminary results assessing the effects of phenolic compounds on acute chlamydial lung infection, and the mechanisms leading to the suppressive effects of luteolin were not studied here. In previous studies, the anti-inflammatory effects of luteolin and quercetin have been reported to be based on the inhibition of proinflammatory cytokine production by suppressing the phosphorylation of nuclear factors such as NF-κB [15,16]. On the other hand, it has been shown that *C. pneumoniae* infection [37], inactivated EB particles [38] and chlamydial heat shock proteins [39] trigger NF-κB activation in

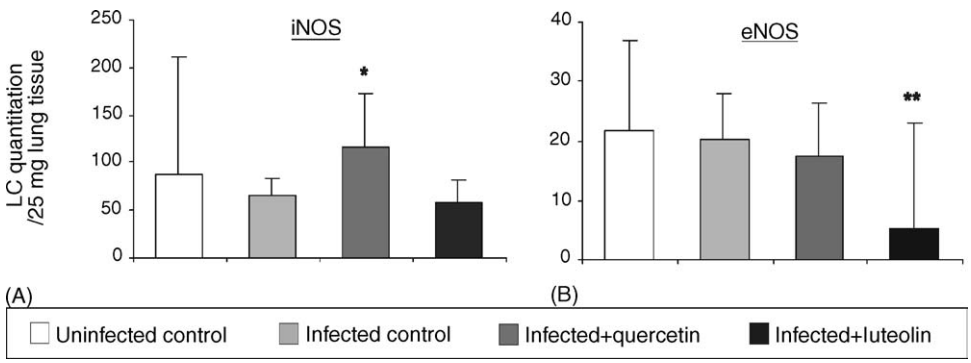

Fig. 5. Expression levels of (A) iNOS and (B) eNOS mRNA in mouse lung tissue. The bars represent geometric means plus 95% upper CI. The quantification results were adjusted for the weight of the tissue taken for mRNA extraction. *Quercetin group vs. infected control group, $p = 0.028$ quercetin group vs. luteolin group $p = 0.019$; **luteolin group vs. infected control group $p = 0.042$.

different cell types. Possibly by suppressing NF-κB-mediated cascades, luteolin was able to diminish the inflammatory reactions induced by chlamydial infection in the present study, though the antioxidative properties of flavonoids may also participate the process. Quercetin, luteolin and octyl gallate are all able to penetrate the cell wall [40], and covalent binding of quercetin on cellular DNA and proteins has been demonstrated earlier [41]. The antibacterial effects of quercetin have been associated at least with the ability to bind bacterial DNA and gyrases [42]. In addition, quercetin and luteolin have been shown to induce apoptosis: luteolin was a significantly more potent inducer than quercetin, and the mitochondrial pathway was suggested to have an important role in the process [43]. The ability of *C. pneumoniae* to inhibit apoptosis of the host cell, which has been reported in several studies, is also mediated via blocking of the mitochondrial pathway [44]. We can speculate that, by abolishing the antiapoptotic effect of chlamydia, these compounds could promote apoptosis of the infected host cells, thus liberating intracellular chlamydial particles vulnerable to the action of host defence.

The effects of quercetin treatment in the present study are contradictory to most of earlier results. Quercetin had no attenuating effect on the lung inflammation, the treatment augmented the load of infective chlamydia in the lungs, and a slight, although not significant, increase in NO levels was detected in serum. On the contrary, Kumar et al. [6] have shown earlier that quercetin reduces oxidative stress with a simultaneous decrease in inflammatory cell infiltration in the lungs of mice inoculated with influenza virus. Quercetin has also been shown to suppresses NOS-mediated NO production after an inflammatory stimulus [19], whereas the present study showed a significant increase in iNOS mRNA expression on day 4 after the infectious challenge in quercetin-treated mice. One possible explanation for these discrepant findings is the higher quercetin dose used in the present study, since earlier studies have shown that, at some point, the increased concentration of either quercetin or luteolin actually has inverse effects on the outcome of infection in vivo [17,45]. In the present study, the compounds were administered intraperitoneally, and the doses were chosen based on the findings of previous in vivo studies in mice [6,17,19] and on the in vitro susceptibility studies in HL cell cultures suggesting the minimal inhibitory concentration (MIC) of *C. pneumoniae* to be 8.8 μmol/l for luteolin, 50 μmol/l for quercetin and 29 μmol/l for octyl gallate [46].

Pharmacokinetics and bioavailability of polyphenolic compounds vary considerably between different groups of flavonoids and also between different forms of a given compound. Most of the flavonoids are found glycosylated in food, and in the case of quercetin, only the glycosylated forms are absorbed whereas absorption of the free, aglycone form has not been detected, as reviewed in [47]. Absorbed compounds are effectively metabolised intracellularly, mostly in the intestine and in the liver but also by other cells [47,48], and only conjugated quercetin metabolites, mainly glucuronides, can be found circulating in the plasma. Fast absorption and appearance of the quercetin metabolites are detected after oral dosing (100 mg) of quercetin glucosides: at 40 min after intake, peak plasma concentration of 2.1 μg/ml was detected [49]. In contrary, absorption of another glycosylate, quercetin rutinoside was much weaker and slower: peak concentration of 0.3 μg/ml was detected 7 h after intake of 200 mg [49]. Unfortunately, very little data exists considering pharmacokinetics and bioavailability of luteolin and octyl gallate. Still, it has been shown that free luteolin aglycone can be found in plasma together with its main metabolite, luteolin monoglucuronide, and luteolin monoglucuronide was also shown to be hydrolysed to free luteolin by the β-glucuronidase of neutrophils at the site of inflammation [50]. Glucuronides or other metabolites of the flavonoids are not as efficient as the unconjugated compounds in view of the beneficial effects of flavonoids detected in vitro [48,51]. The bioavailability of luteolin at the inflammatory foci might, therefore, be better than that of quercetin, which would explain at least partly the difference between the compounds seen in our study. Concentrations of quercetin and luteolin aglycones in the mice serum samples at 1 h after an intraperitoneal injection of the compounds were analysed in the present study, but no detectable concentrations were found (results not shown).

Tissue distribution of flavonoids after intake in humans is not known, however, as reviewed by Manach et al. [47], based on two reports studying a specific tissue, plasma concentrations may not correlate with the flavonoid concentration in a certain tissue. A study in mice with a [^{14}C]-biolabelled polyphenolic compound *trans*-resveratrol showed that at 3 h after oral intake, radioactivity was present with the highest amounts in duodenum, kidney, lung, liver and spleen and also in colon, heart, brain and testis [52], thus indicating a wide distribution at least in mice. Also relatively low and steady radioactivity in blood was detected in this study, although significant changes in tissue concentrations were seen. One functional site for flavonoids may also be endothelium, since it has been shown that active and rapid transport system exists in aortic endothelial cells for the uptake of a flavonol morin [53]. Interestingly, Alvesalo et al. [46] have shown that preincubation of HL-cells with phenolics quercetin, rhamnetin, morin and octyl gallate, i.e. the presence of a compound prior to inoculation, was able to significantly decrease the formation of chlamydial inclusions in the cells.

The beneficial antichlamydial and anti-inflammatory effects of luteolin treatment on acute *C. pneumoniae* lung infection in vivo were demonstrated in this study. Luteolin was able to inhibit the presence of infective bacteria in lung tissue and to suppress lung inflammation induced by intranasal *C. pneumoniae* infection. Whether the spread

of chlamydia from lungs to other tissues can be attenuated by this treatment as well should be assessed in the further studies. Assuming the pathogen burden as a risk factor for atherosclerosis [28], by suppressing the inflammatory reactions elicited by an infection, luteolin and other flavonoids could have an effect on the atherosclerotic process. Our findings are preliminary results on the possible beneficial effects of flavonoid compounds on *C. pneumoniae* infection in an animal model. Further studies should clarify whether oral or alternatively topical administration by inhalation, as proposed by Yamazaki et al. [54], of these compounds could influence the course of acute infection and, even more importantly, the course of chronic infection in mice and humans.

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