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## ROLE OF DIETARY ANTIOXIDANTS IN HUMAN METAPNEUMOVIRUS INFECTION

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### Summary

Human metapneumovirus (hMPV) is a major cause of respiratory tract infections in children, elderly and immunocompromised hosts, for which no vaccine or treatment are currently available. Oxidative stress and inflammatory responses represent important pathogenic mechanism(s) of hMPV infection. Here, we explored the potential protective role of dietary antioxidants in hMPV infection. Treatment of airway epithelial cells with resveratrol and quercetin during hMPV infection significantly reduced cellular oxidative damage, inflammatory mediator secretion and viral replication, without affecting viral gene transcription and protein synthesis, indicating that inhibition of viral replication occurred at the level of viral assembly and/or release. Modulation of proinflammatory mediator expression occurred through the inhibition of transcription factor nuclear factor (NF)- $\kappa$ B and interferon regulatory factor (IRF)-3 binding to their cognate site of endogenous gene promoters. Our results indicate the use of dietary antioxidants as an effective treatment approach for modulating hMPV induced lung oxidative damage and inflammation.

### Keywords

Human metapneumovirus; resveratrol; quercetin

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Human metapneumovirus (hMPV) is a paramyxovirus identified a little over a decade ago (van den Hoogen *et al.*, 2001), with genome closely resembling that of respiratory syncytial virus (RSV), with few variances in gene order and absence of the non-structural genes NS1 and NS2 (Schildgen *et al.*, 2011). hMPV is a major cause of lower respiratory tract infections, such as bronchiolitis and pneumonia, in children, elderly and immunocompromised hosts, with an economic impact in the pediatric population similar to that of influenza virus (Boivin *et al.*, 2007; Edwards *et al.*, 2013; Jartti *et al.*, 2002).

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Several viral infections, including human immunodeficiency virus (HIV), Hepatitis B and C, rhinovirus, influenza and RSV, have been shown to induce reactive oxygen species (ROS) formation in a variety of cell types (Bao *et al.*, 2008b; Casola *et al.*, 2001; Peterhans, 1997). ROS play an important role in cellular signaling, leading to the expression of proinflammatory mediators, and uncontrolled ROS generation is associated with cellular damage, a condition known as oxidative stress (Cienciewicki *et al.*, 2008; Djordjevic, 2004). Many features of influenza- and RSV-mediated lung disease including inflammation, airway hyperreactivity, and epithelial cell damage are due to oxidative stress (Castro *et al.*, 2006; Vlahos *et al.*, 2011). Recently, we have shown that hMPV infection of airway epithelial cells induces proinflammatory gene expression through activation of transcription factors nuclear factor kappa B (NF- $\kappa$ B) p65 subunit and interferon regulatory factor (IRF)-3 (Bao *et al.*, 2007; Bao *et al.*, 2008a; Kolli *et al.*, 2012). hMPV infection also induces oxidative stress by progressive decreasing gene expression and protein levels of antioxidant enzymes (AOEs) such as superoxide dismutase 3, catalase, glutathione S-transferase, both *in vitro* and *in vivo* (Bao *et al.*, 2008b; Hosakote *et al.*, 2011). We also found that hMPV infection reduced nuclear translocation of the transcription factor NF-E2-related factor 2 (Nrf2) (Komaravelli & Casola, 2014), a major regulator of AOE gene expression (Jaiswal, 2004).

Dietary supplements like resveratrol and quercetin, which possess anti-oxidant and anti-inflammatory properties, have been beneficial in modulating oxidative stress and inflammation against a variety of diseases (Angeloni *et al.*, 2007; Baur & Sinclair, 2006). Resveratrol is a non-flavonoid polyphenol abundant in skin of red grapes, peanuts and red wine (Pollack & Crandall, 2013). Quercetin is a flavonoid abundant in apple skins, onion peels, peppers, certain berries and green tea (Boyer *et al.*, 2004). Both these dietary supplements possess anti-carcinogenic, anti-inflammatory, antioxidant and antiviral properties (Baur & Sinclair, 2006; Campagna & Rivas, 2010; Chirumbolo, 2010). In respiratory viral infections, resveratrol and quercetin treatment had a protective effect against influenza infection [reviewed in (Uchide & Toyoda, 2011)], while quercetin protected against rhinovirus both *in vitro* and *in vivo* (Ganesan *et al.*, 2012). However, the role of these molecules in the context of hMPV infection has never been investigated.

To investigate the effect of resveratrol and quercetin treatment on hMPV-induced cellular oxidative damage and inflammation response, A549 cells (alveolar type II cancerous cell line) were infected with hMPV at multiplicity of infection (MOI) of 1 as previously described (Bao *et al.*, 2008b), in the presence or absence of various doses of resveratrol (10–50  $\mu$ M in DMSO) or quercetin (1–10  $\mu$ M in DMSO). Culture supernatant was harvested at 24h post-infection (p.i.) to measure the oxidative stress damage marker F<sub>2</sub> 8-isoprostane, using a competitive enzyme immunoassay from Cayman Chemical (Ann Arbor, MI), and proinflammatory markers CXCL8 (IL-8) and CCL5 (RANTES). We also tested for changes in viability due to antioxidant treatment by Trypan blue exclusion and there was no effect of both compounds on uninfected cells. hMPV infection was associated with significant oxidative damage in A549 cells, as evident by increased release of 8-isoprostane, and with a significant increase in the secretion of the proinflammatory cytokines IL-8 and RANTES. Both resveratrol and quercetin treatment significantly reduced hMPV-induced 8-isoprostane generation, to levels comparable to mock-infected (Ctrl) cells (Fig. 1A), as well as chemokines IL-8 and RANTES (Fig. 1B). Mock infected cells represent uninfected cells

treated as infected cells, with media contains lower serum concentrations (2%) and an amount of sucrose equivalent to the one present in the viral inoculum (Ueba, 1978). We also assessed other cytokine and chemokine production in the cell supernatant of the highest effective concentrations of resveratrol and quercetin using the Bio-Plex Cytokine Human Multi-Plex Assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. hMPV infection was associated with a significant increase in the secretion of the proinflammatory cytokines IL-1 $\alpha$ , IL-6 and TNF- $\alpha$ , and the chemokines CXCL10 (IP-10) and CCL4 (MIP-1 $\beta$ ). Both resveratrol and quercetin treatment significantly reduced these cytokine and chemokine secretion from infected cells (Fig.1C).

To investigate the effect of these dietary antioxidants on viral replication, A549 cells were infected with hMPV at MOI of 1 in the presence or absence of resveratrol or quercetin and harvested at 24h p.i. to prepare total RNA by ToTALLY RNA kit (cat AM1910, Ambion, Life technologies, Austin TX) and analyze the absolute copy number of the hMPV gene N by quantitative real-time PCR (qRT-PCR). Neither resveratrol nor quercetin treatment had an effect on hMPV N gene transcription (Fig.2A). To investigate their effect on hMPV protein expression, total cell lysates were prepared in RIPA buffer and Western blot analysis was performed using a polyclonal rabbit anti-hMPV antibody, as described in (Bao *et al.*, 2013). Again, there was no significant change in the amount of hMPV total protein expression in resveratrol or quercetin treated compared to untreated, infected cells (Fig.2B). To determine whether dietary antioxidant treatment would affect later steps of viral replication, production of viral infection particles was determined by plaque immunoassay, as previously described (Guerrero-Plata *et al.*, 2005). Resveratrol and quercetin treatment of A549 cells during hMPV infection was associated with a much lower viral titer, compared to untreated, infected cells (Fig.2C), suggest that these antioxidants interferes with viral assembly and formation of mature virus. In resveratrol treated cells, the amount of infectious viral particles reduced from almost a million/ml down to 50,000 and quercetin treatment reduced viral particles to little above 100,000. Resveratrol treatment has been shown to be protective against influenza (Palamara *et al.*, 2005), RSV (Liu *et al.*, 2014;Xie *et al.*, 2012;Zang *et al.*, 2011), herpes simplex virus 1 and 2 (Docherty *et al.*, 1999;Faith *et al.*, 2006), Epstein-Barr virus (De *et al.*, 2011), human cytomegalovirus (Evers *et al.*, 2004), varicella-zoster virus (Docherty *et al.*, 2006) and polyomavirus (Berardi *et al.*, 2009) infections, however it enhanced viral replication of Hepatitis C virus *in vitro* (Nakamura *et al.*, 2010) and aggravated Theiler's murine encephalomyelitis virus-induced demyelinating disease in mice (Sato *et al.*, 2013). Resveratrol treatment inhibition of influenza A virus replication was due to reduced expression of viral proteins through inhibition of a PKC-dependent pathway (Palamara *et al.*, 2005). Resveratrol treatment has also been shown to suppress RSV replication in airway epithelial cells (Liu *et al.*, 2014;Xie *et al.*, 2012;Zang *et al.*, 2011) and reduce RSV-induced production of cytokines IFN- $\gamma$  and IL-6 through inhibition of TLR3/TRIF signaling (Liu *et al.*, 2014;Xie *et al.*, 2012;Zang *et al.*, 2011), although no significant effect was observed on the production of chemokines, such as IL-8. Quercetin has been shown to inhibit rhinovirus and influenza replication *in vitro* and *in vivo*, by affecting different stages of virus life cycle, and to reduce proinflammatory mediator production (Ganesan *et al.*, 2012;Kim *et al.*, 2010). There is only one study investigating the effect of dietary flavonoids, including quercetin, on the infectivity and replication of RSV, in which

quercetin did not affect the ability of the virus to infect the cells or replicate (Kaul *et al.*, 1985). We observed a similar result when we tested quercetin in RSV-infected cells at the same concentration that was effective in reducing hMPV replication (data not shown), suggesting that quercetin antiviral effect is virus-specific.

We have previously shown that hMPV activates the RIG-I-MAVS signaling pathway in airway epithelial cells, leading to the activation of NF- $\kappa$ B and IRF-3 and the corresponding expression of proinflammatory and antiviral molecules (Liao *et al.*, 2008). To investigate the mechanism(s) by which resveratrol and quercetin decreased hMPV-induced proinflammatory mediator production, we assessed NF- $\kappa$ B/p65 and IRF-3 activation. A549 cells infected with hMPV at MOI of 1 in the presence or absence of these antioxidants were harvested at 24h p.i. to prepare nuclear protein by hypotonic/nonionic detergent lysis, as previously described (Bao *et al.*, 2008a;Schreiber *et al.*, 1989). NF- $\kappa$ B/p65 and IRF-3 nuclear levels were assessed by western blot analysis using specific antibodies anti-p65 (#4764, Cell Signaling, Boston, MA) and anti-IRF-3 (sc-9082, Santa Cruz Biotechnology, Dallas, TX). hMPV infection resulted in significant p65 and IRF-3 nuclear translocation, compared to mock-infected cells, which was not affected by resveratrol or quercetin treatment (Fig.3A). To determine whether antioxidants influenced occupancy of NF- $\kappa$ B/p65 and IRF-3 of their cognate sites of endogenous gene promoters, we performed chromatin immunoprecipitation (ChIP) and quantitative genomic PCR assays (Q-gPCR) (Tian *et al.*, 2012). For ChIP assays, we used ChIP-IT Express kit from Active Motif (53008 Carlsbad, CA), following manufacturer instruction with some modifications. Briefly, cells infected with hMPV for 24h in the presence or absence of resveratrol or quercetin were fixed with 2mM disuccinimidyl glutarate and formaldehyde at room temperature, nuclei were isolated, sheared by sonication, and 20  $\mu$ g of sheared chromatin was immunoprecipitated with 5  $\mu$ g of ChIP grade anti-NF- $\kappa$ B/p65 (ab7970, abcam, Cambridge, MA) or -IRF-3 antibodies (sc-9082x, Santa Cruz Biotechnology, Dallas, TX), and magnetic beads conjugated with protein G at 4°C overnight. Immunoprecipitation with IgG antibody was used as negative control. Chromatin was reverse cross linked, eluted from magnetic beads, and purified. Q-gPCR was done by SyBR green based real-time PCR using the following primers spanning the IL-8 gene NF- $\kappa$ B promoter site: forward-AGGTTTGCCCTGAGGGGATG and reverse-GGAGTGCTCCGGTGGCTTTT, or the RANTES gene ISRE promoter site: forward-AGCGGCTTCTGCTCTCTGA and reverse- CAGCTCAGGCTGGCCCTTTA. Total input chromatin DNA for immunoprecipitation was included as positive control for PCR amplification. hMPV infection dramatically increased NF- $\kappa$ B/p65 binding to the IL-8 promoter and IRF-3 binding to the RANTES promoter, which was significantly reduced by both resveratrol and quercetin treatment (Fig.3B), leading to reduced expression of cytokines and chemokines. NF- $\kappa$ B must undergo a variety of post-translational modification, including phosphorylation and acetylation, to achieve its full biological activity (Chen & Greene, 2004), (Chen *et al.*, 2005). Acetylation of specific lysine residues has been shown to modulate distinct NF- $\kappa$ B functions (Chen & Greene, 2004;Schmitz *et al.*, 2004). A possible mechanism by which resveratrol could affect hMPV-induced p65 activation is by inducing Sirtuin1 (SIRT1) (Park *et al.*, 2012), which has histone deacetylase activity. SIRT1 has been shown to antagonize NF- $\kappa$ B activation (Kauppinen *et al.*, 2013), and reduced SIRT1 activity by HIV TAT protein was associated with increased binding of NF- $\kappa$ B to the promoter of

proinflammatory genes (Blazek & Peterlin, 2008; Kwon *et al.*, 2008; Yeung *et al.*, 2004). Reduced SIRT1 activity has also been shown to be associated with increased activity of IRF-3 in adipose inflammation (Gillum *et al.*, 2011), suggesting an antagonistic crosstalk between SIRT1 and IRF-3 also.

In conclusion, we found that resveratrol and quercetin treatment significantly reduced hMPV-induced oxidative damage, inflammatory responses and viral replication, suggesting their potential use as prophylactic and possibly therapeutic intervention for modulating hMPV-associated inflammatory lung disease, which deserve further testing *in vivo*, in an animal model of infection for example.

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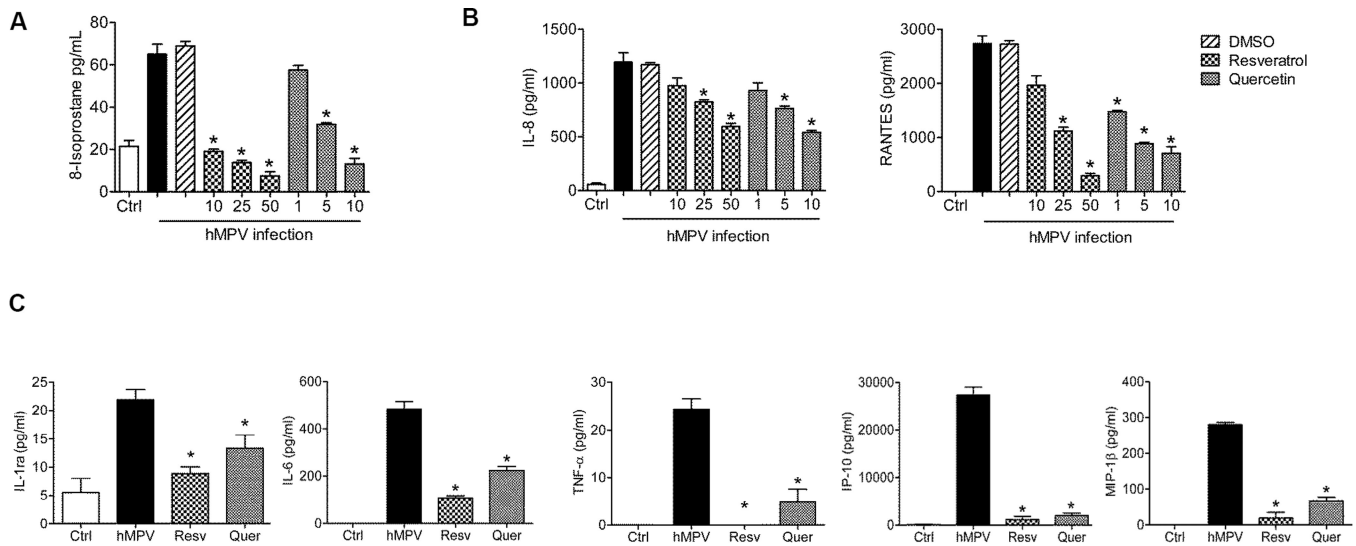
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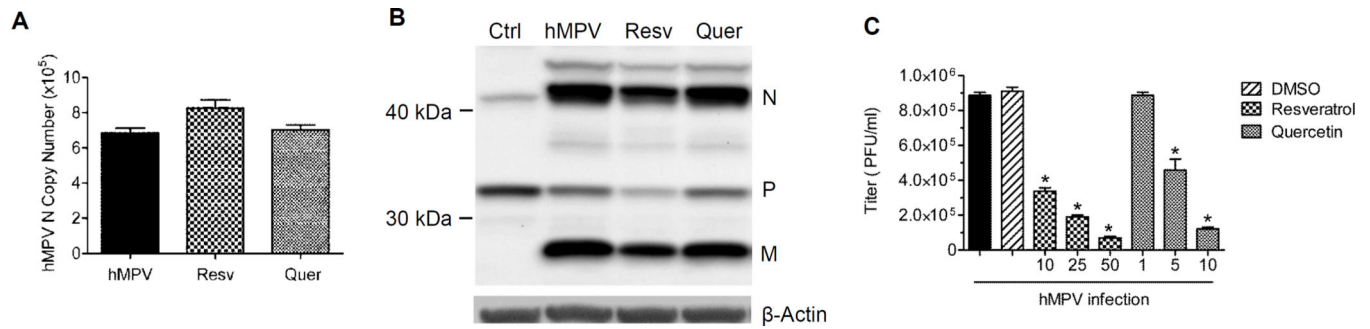
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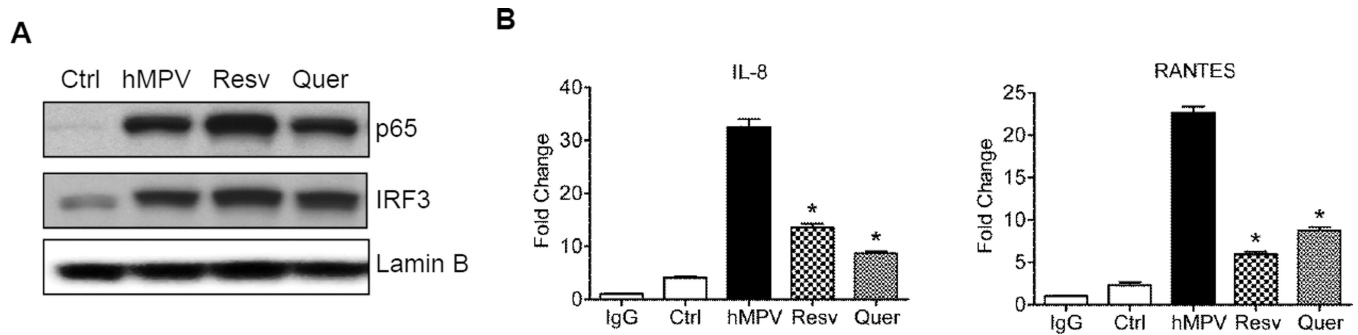
**Figure 1. Effect of resveratrol and quercetin on oxidative stress and inflammatory mediator production**

A549 cells were either mock-infected (Ctrl) or infected with hMPV in the presence or absence of DMSO at MOI of 1 for 24h and treated or untreated with resveratrol (10–50 μM in DMSO) or quercetin (1–10 μM in DMSO). Culture supernatants were harvested to measure (A) oxidative stress marker 8-isoprostane; (B) IL-8 and RANTES (C) Since DMSO did not have any significant effect on hMPV infection, we did not include vehicle control in further experiments. Supernatants from A549 cells either uninfected (Ctrl) or infected with hMPV and treated or untreated with resveratrol (50 μM) or quercetin (10 μM) were measured for cytokines and chemokines. Results are expressed as mean ± standard error and are representative of three independent experiments. \**P* < 0.05 relative to hMPV-infected cells.



### Figure 2. Effect of resveratrol and quercetin on viral replication

A549 cells were either mock-infected (Ctrl) or infected with hMPV at MOI of 1 for 24h in the presence or absence of 50  $\mu$ M resveratrol or 10  $\mu$ M quercetin, and harvested to prepare (A) total RNA to quantify RSV N gene expression by qRT-PCR; and (B) total cell lysates for western blot analysis for hMPV viral protein expression. For loading controls, membrane was stripped and reprobbed with anti  $\beta$ -actin antibody; (C) A549 cells infected with hMPV at MOI of 1 for 24h and treated or untreated with various doses of resveratrol (10 – 50  $\mu$ M in DMSO) or quercetin (1 – 10  $\mu$ M in DMSO) or vehicle alone were harvested to measure viral titer by plaque assay. Results are expressed as mean  $\pm$  standard error and are representative of three independent experiments. \* $P$  < 0.05 relative to hMPV-infected cells.



**Figure 3. Effect of resveratrol and quercetin on transcription factor activation**

A549 cells were infected with hMPV at MOI of 1 for 24h in the presence or absence of 50  $\mu$ M resveratrol or 10  $\mu$ M quercetin. (A) Nuclear proteins were analyzed for NF- $\kappa$ B/p65 and IRF-3 levels by Western blot. For loading controls, membranes were stripped and reprobed with anti lamin B antibody. (B) ChIP-QgPCR analysis of p65 occupancy of the IL-8 gene promoter NF- $\kappa$ B site (left panel) and IRF-3 occupancy of the RANTES gene promoter ISRE site (right panel). Data shown is representative of three independent experiments. \* $P < 0.05$  relative to 24h hMPV infected cells.