The Protective Effects of a Polyphenol-Enriched Protein Powder on Exercise-Induced Susceptibility to Virus Infection

By: **Dru A. Henson**, Maryam Ahmed, **Matthew C. Sanderson**, David C. Nieman, Nicholas D. Gillitt & Mary Ann Lila

Abstract

Prolonged and intensive exercise induces transient immunosuppression and is associated with an increased risk and severity of infections. The goal of this study was to characterize the antiviral and antibacterial properties of the bioactive metabolites of a blueberry–green tea-polyphenol soy protein complex (PSPC) in the serum of supplemented subjects during a 3-day intensified training period. Long-distance runners, randomly divided into two groups, ingested 40 g/day PSPC or placebo (soy protein and colorings) for 17 days, with a 3-day running period inserted at day 14. Blood serum samples were collected pre-14 days and post-14 days supplementation, and immediately and 14 h after the third day of running. The post-exercise serum from both groups significantly promoted the growth of Escherichia coli and Staphylococcus aureus in culture by 20–70%, but returned to normal levels following recovery. Furthermore, the serum from subjects ingesting PSPC did not display antibacterial properties at any time point. In contrast, there was a significant difference in the ability of serum from PSPC-supplemented versus placebo-supplemented athletes to protect cells in culture from killing by vesicular stomatitis virus following strenuous exercise. In addition, the serum of subjects who ingested PSPC significantly delayed an exercise-induced increase in virus replication. These results indicate that polyphenol complexes containing blueberry and green tea have the potential to protect athletes from virus infections following rigorous exercise.

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INTRODUCTION

Polyphenols are abundant micronutrients in our diet and are widespread constituents of fruits, vegetables, cereals, chocolates and beverages such as tea, coffee, beer and wine. Numerous studies have revealed that polyphenols exert beneficial effects on human health, including antioxidant, anti-inflammatory and anti-cancer activity (Gonzalez-Vallinas *et al.*, 2013; Khurana *et al.*, 2013; Landete, 2012). Furthermore, polyphenols derived from various sources have been reported to exert activity against a variety of infectious organisms such as respiratory, sexually transmitted and food-borne viruses, and pathogenic bacterial strains (Andrae-Marobela *et al.*, 2013; Daglia, 2012; Fioravanti *et al.*, 2012; Li *et al.*, 2013). However, much of the evidence on the prevention of diseases and infections by polyphenols is derived from *in vitro* or animal studies that are often performed with doses much higher than those available to humans. Furthermore, polyphenols are extensively metabolized by intestinal and hepatic enzymes and by the intestinal microflora to generate circulating conjugated derivatives that are made available to target tissues (Del Rio *et al.*, 2013). Therefore, *in vitro* studies with polyphenolic extracts may fail to accurately evaluate the effects of the various derivatives in humans.

The goal of the current study was to determine whether the serum of subjects ingesting a high-dose polyphenol supplement would provide protective activity against viral and bacterial infections following a 3-day intensified training regimen. During strenuous exercise, there is a dramatic increase in reactive oxygen species production resulting in oxidative stress (Powers and Jackson, 2008). Exhaustive exercise also induces structural damage to muscle cells, inflammation and immune dysfunction. Transient immunosuppression induced by prolonged, intense exercise is reported to increase the risk of acquiring respiratory tract infections in humans (Martin *et al.*, 2009; Nieman, 1994). Therefore, there is great interest in evaluating the anti-microbial effects of polyphenols during strenuous exercise-induced immune dysfunction in humans.

A number of epidemiological studies have attempted to determine the effects of specific intensities and durations of exercise on the incidence of upper respiratory tract infections in humans (Ekblom *et al.*, 2006; Nieman *et al.*, 1989; Nieman *et al.*, 1990). However, because of ethical constraints, there are limited studies that have evaluated whether polyphenolic compounds exert protective effects against infectious organisms during strenuous exercise in humans. We have developed *in vitro* assays to evaluate the antiviral and antibacterial activity in the serum of individuals supplemented with a blueberry and green tea-polyphenol-rich soy protein-based

complex (PSPC). This study expands on the work done by Nieman *et al.* in which they determined that 17 days of supplementation with the PSPC compound caused a distinct gut-derived phenolic signature in long-distance runners following a 3-day period of intensified running (Nieman *et al.*, 2013). However, PSPC supplementation did not alter conventional biomarkers for inflammation and oxidative stress at any point during the study. By testing the effect of PSPC supplementation on the growth properties of specific bacteria and viruses and their impact on cell killing, we determined that serum factors circulating after ingestion of green tea and blueberries have the capacity to exert antiviral activity.

MATERIALS AND METHODS

Subjects and research design. Subject recruitment was conducted as described previously (Nieman *et al.*, 2013). Subjects were recruited by the Human Performance Laboratory Research Manager, and included 38 healthy, non-smoking long-distance male or female runners ages 19–45 years who regularly competed in marathon and half-marathon road races and were capable of running 2.5 h at high intensity on laboratory treadmills. During the study, subjects consented to train normally, maintain weight and avoid the use of medications known to affect inflammation and immune function for the duration of the study. All vitamin and mineral supplements above 100% the U.S. Daily Value were also avoided. Runners ($N = 35$) were randomized by the research manager and divided into the PSPC ($N = 17$) or placebo groups ($N = 18$), with supplements administered over a 17-day period using double-blinded methods and a parallel group design. All other investigators, study personnel and subjects were blinded to the type of supplement used by the two groups during the study. All subjects signed informed consent forms, and all study procedures were approved by the Institutional Review Board at Appalachian State University (ASU). Data were analyzed from subjects completing all aspects of the study ($N = 16$ PSPC, $N = 15$ placebo). The study was conducted during the winter/spring of 2012 at the ASU Human Performance Laboratory at the North Carolina Research Campus in Kannapolis, NC.

Supplementation complex and procedures. Green tea extract (product# Std +101) was purchased from Finlay Tea Solutions US Inc. (Florham Park, NJ, USA). Liquid blueberry pomace water extract was a gift from Milne Fruit (Prosser, WA, USA). Soy protein isolate (SPI) was obtained from Archer Daniels Midland (Ardex F, ADM, Decatur, IL, USA). All clinical trial study materials were provided by Nutrasorb LLC (North Brunswick, NJ, USA), and supplements prepared by the Dole Nutrition Research Institute. Blueberry polyphenol soy protein complex (22 kg) and green tea-polyphenol soy protein complex (8 kg) were produced individually as previously described (Nieman *et al.*, 2013), and then blended to obtain a 3:1 blueberry–green tea PSPC. The total polyphenolic levels and individual catechin contents remained stable for greater than 12 months storage at 5 °C in the dark due to the stable protein polyphenolic matrix (Nieman *et al.*, 2013). Placebo was prepared from SPI, with non-polyphenolic food colorings (mixture of FD&C Blue #1 and FD&C Re #40) added to approximate the purple hue of PSPC.

Supplementation took place over a 17-day period, including a 14-day pre-exercise period, followed by a 3-day intensified exercise period. Subjects were randomized to PSPC or placebo (parallel group design, double-blinded treatments), and consumed 20 g mixed in 237 mL water in the morning, and then 20 g again at lunch (thus 40 g/day). Overall, the effective daily dose of PSPC (40 g) corresponded to 2136 mg Gallic Acid Equivalents (GAEs) or the equivalent of 3 cups of blueberries and 1.5 cups of brewed green tea (Nieman *et al.*, 2013). Compliance to the supplementation regimen was checked through email messages and during the post-supplementation lab visits.

Exercise sessions. Polyphenol soy protein complex and placebo groups did not differ significantly in age (33.7 ± 1.7 and 35.2 ± 2.2 years, respectively, $P = 0.593$) and VO_{2max} (57.2 ± 1.8 and 54.3 ± 1.6 years, respectively, $P = 0.232$). Subjects in both groups trained normally during the 2-week supplementation (average 49.0 ± 2.7 km/week) and then participated in a 3-day period of intensified exercise (Fig. 1). In the morning of the first intensified exercise session, subjects consumed the normal supplement portion (20 g PSPC or placebo in 237 mL water) and breakfast (ad libitum). A standardized meal consisting of Boost Plus at 10 kcal/kg was ingested at 12:00 noon. Boost Plus is manufactured by Nestle (Vevey, Switzerland) and has an energy density of 1.5 kcal/mL (50% energy as carbohydrate, 35% fat and 15% protein). The normal lunchtime PSPC or placebo supplement was delayed until after the blood draw at 2:30 PM. Subjects reported to the Human Performance Laboratory at 2:30 PM and

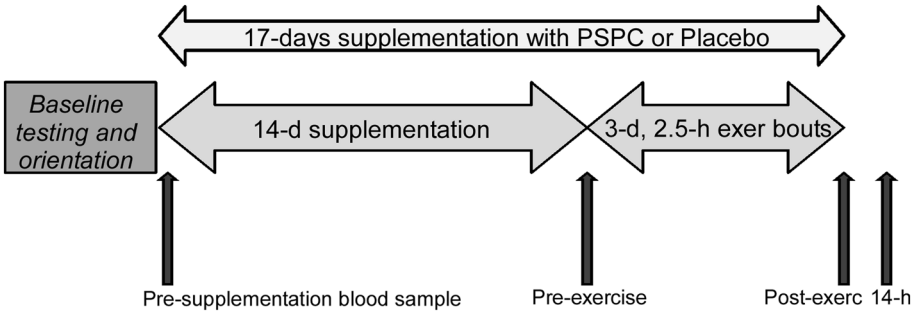

Figure 1. Research design. Thirty-one randomized runners ingested polyphenol soy protein complex (PSPC) ($N = 16$) or placebo ($N = 15$) for 2 weeks and then completed 3 consecutive days of intense bouts of 2.5 h exercise at 90–75% VO_{2max}. Blood samples were collected as indicated.

provided blood samples in a rested, seated position. Subjects next ingested the PSPC or placebo supplement dose. At 3:00 PM, subjects ran on treadmills for 2.5 h at approximately 70% VO_{2max}. Water was given ad libitum throughout the 2.5-h exercise bouts, with no other beverage or food allowed. Subjects ingested another 20 g dose of PSPC or placebo mixed in water after 1 hour of exercise. Heart rate, rating of perceived exertion and distance run were recorded every 30 min during the bout, with oxygen consumption and ventilation measured after 1 h of exercise (Cosmed FitMate metabolic system, Rome, Italy). No group differences in metabolic measures were found for the 2.5-h exercise periods over the 3-day intensified exercise period. For PSPC and placebo groups, 3-day averages during 7.5 h running on level treadmills for heart rate (HR) were 152 ± 3.8 ($82.1 \pm 0.9\%$ HR_{max}) and 151 ± 2.5 bpm ($84.2 \pm 0.9\%$ HR_{max}), respectively ($P = 0.930$), oxygen consumption (VO_2) were 37.8 ± 1.0 ($66.5 \pm 1.8\%$ VO_{2max}) and 36.5 ± 0.8 mL kg^{-1} min^{-1} ($67.8 \pm 2.0\%$ VO_{2max}), respectively ($P = 0.326$), and distances run were 25.3 ± 0.7 and 25.0 ± 0.5 km/day, respectively ($P = 0.777$). Subjects repeated this schedule for the next 2 days but without pre-exercise blood draws. Blood samples were taken immediately following the third exercise bout on the third day and 14-h post-exercise the following morning. In total, four samples of blood were collected from each subject (pre-supplementation, pre-exercise, post-3 day exercise and 14-h recovery) as indicated in Fig. 1. Blood was collected in serum separator tubes and allowed to clot for 30 min. Following centrifugation, serum was aliquoted and stored at −80 °C until use.

Antibacterial activity. Serum samples from PSPC-supplemented and placebo-supplemented subjects were assessed for their ability to affect the growth of select bacteria. Serum samples at each time point were heat-inactivated for 30 min at 56 °C (HI-serum) to prevent the complication of complement-mediated cell lysis. HI-serum samples were diluted at a 1:2 ratio with LB bacterial nutrient broth (Fisher Scientific) and incubated with 20 µL of overnight cultures of *Escherichia coli* (*E. coli*) strain K-12 or coagulase-positive *Staphylococcus aureus* (*S. aureus*), a BSL2 pathogen, both obtained from Carolina Biological Supply Company (Burlington, NC, USA). Controls included serum-alone samples, bacteria-alone samples (growth control), nutrient broth samples (sterility control) and bacteria grown in the presence of 128 µg/mL of ampicillin (obtained from Fischer Scientific). All experimental and control cultures were incubated at 37 °C for 24 h, at which time samples were carefully re-suspended and transferred to a 96-well dish. Absorbance readings at 600 nm were measured using the Molecular Devices SoftMax Pro 5 microplate reader. Data were quantitated by dividing the experimental samples (experimental minus serum alone) by the growth control samples (growth control minus sterility control). These values were then normalized to the pre-supplementation values in order to more clearly observe changes following supplementation, exercise and recovery periods.

Cells and virus. HeLa cervical cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% fetal bovine serum (FBS). HeLa cells were used for our study because of their robust growth properties and their susceptibility to infection by vesicular stomatitis virus (VSV).

Vesicular stomatitis virus is a prototype of negative strand RNA viruses such as rabies virus and Ebola virus. It is highly cytopathic for cells from a variety of different species, including neurons and respiratory epithelial cells, thus making it a desirable model for the study of pathogenic viruses. Recombinant wild-type VSV (rwt virus) contains genes from several wt strains of VSV. Rwt virus and recombinant VSV expressing EGFP (green fluorescent protein) as a foreign gene (rwt-GFP virus) have been described previously (Whitlow *et al*., 2006). Virus stocks were prepared in HeLa cells using methods described previously (Kopecky *et al*., 2001). For all virus infections, HeLa cells were infected in DMEM with 7.5% FBS at a multiplicity of infection (MOI) of 3 PFU/cell.

Antiviral activity. To investigate whether serum from test subjects exhibited antiviral activity, the ability of VSV to kill permissive cells in the presence of various concentrations of serum was determined by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. Specifically, HeLa cells, susceptible to infection by VSV, were grown in 96-well dishes (in DMEM plus 7.5% FBS) until they reached approximately 70–80% confluency. At this time, cells were incubated with HI-serum at a serum to media ratio of 1:2. At various times post-incubation (0 and 4 h), cells were infected with rwt virus at a MOI of 3 plaque-forming unit (pfu)/cell. Cells were assessed for viability using MTT assay (MTT Cell Proliferation Kit, Roche Diagnostics) at 24 and 48 h post-infection. Controls included incubation of cells with serum alone (serum-alone samples) to measure potential cytotoxic effects of the serum, virus alone (virus-alone samples) to determine the cytotoxic effect of virus, and untreated and uninfected cells (mock) as background controls. Data were quantitated by dividing the values obtained from each sample by the mock value. All data were then normalized to the serum-alone data to adjust for differences in the serum responses between subjects. As defined previously, these values were then normalized to the pre-supplementation values.

Virus replication. To determine the effect of serum from test subjects on the replication capacity of VSV, HeLa cells were grown in 6-well dishes until they reached 70–80% confluency. As described earlier, cells were incubated with HI-serum at a serum to media ratio of 1:2. At 4 h post-incubation, cells were infected with rwt-GFP at a MOI of 3 pfu/cell. At 6 and 12 h post-infection, cells were washed, harvested in PBS and subjected to flow cytometry to measure the fluorescence of GFP within cells. Two parameters were measured: (1) the percentage of cells expressing GFP, as an indication of the number of cells supporting virus replication, and (2) the geometric mean fluorescence, measuring the degree of replication within cells. All data were normalized to the pre-supplementation values.

Statistical methods. All data are expressed as mean ± standard error. Data were compared between groups at each time point using Student's *t*-test, with comparisons between time points within a group compared using paired *t*-test.

RESULTS

Effect of polyphenol soy protein complex on bacterial growth

Several studies have investigated the effects of phytochemicals found in blueberries and green tea on inhibition of pathogenic bacteria. Various berry extracts have been shown to inhibit the growth of uropathogenic bacteria such as *Helobacter pylori* and *E. coli* (Guay, 2009; Roopchand *et al.*, 2012; Zafra-Stone *et al.*, 2007). The main constituent of green tea, epigallocatechin-3-gallate (EGCG), has been reported to have anti-infective properties against *S. aureus* strains using multiple mechanisms (Steinmann *et al.*, 2013). Data in Fig. 2 show that there was no significant difference in the growth of *E. coli* (Fig. 2A) or *S. aureus* (Fig. 2B) in the presence of serum from placebo-supplemented versus PSPC-supplemented subjects. However, we observed a significant exercise-induced increase in bacterial growth of *E. coli* in both the placebo (58% increase, $P = 0.005$) and PSPC (71% increase, $P = 0.006$) groups, and *S. aureus* in the placebo group (23% increase, $P = 0.033$), which decreased during the recovery period. These data indicate that intense and prolonged exercise leads to increased susceptibility to bacterial infection that is not ameliorated by PSPC.

Antiviral effects of polyphenol soy protein complex supplementation

In addition to negatively impacting the growth of bacteria, polyphenols have also been reported to possess antiviral activity. The antiviral activities of ECGC with different modes of action have been demonstrated for important human pathogens such as HIV and influenza virus (Andrae-Marobela *et al.*, 2013; Fioravanti *et al.*, 2012). In addition, *in vitro* studies have shown that ECGC at physiological concentrations interferes with the replication cycles of DNA viruses like hepatitis B virus, herpes simplex virus and adenovirus (Steinmann *et al.*, 2013). In order to measure the antiviral activity in serum from PSPC-supplemented and placebo-supplemented individuals, we exposed HeLa cells to serum and evaluated its ability to protect cells against killing by VSV. To determine the dose of VSV used for the experiments, HeLa cells were infected with rwt virus at MOIs of 0.3 and 3 pfu/cell, and cell viability was measured at 24 and 48 h post-infection (Fig. 3A). At the lower dose of virus (MOI 0.3 pfu/cell), HeLa cells succumbed to killing by rwt virus only when infected for 48 h. However, when infected at a MOI of 3 pfu/cell, cells were highly susceptible to the cytopathic effect of VSV such that less than 20% of cells remained viable by 24 h. For the subsequent experiments (Fig. 3C and D), we chose to infect cells at an MOI of 3 pfu/cell for 24 h in order to measure any potential antiviral activity in serum samples.

There was no difference in the effect of serum alone from PSPC- ($N = 16$) versus placebo-supplemented ($N = 15$) subjects on cell viability (Fig. 3B). Furthermore, serum alone did not inhibit the viability of HeLa. In fact, there was a slight increase in cell viability following exercise in both sample groups. To measure the effect of serum on the viability of cells following virus infection, data were normalized to serum-alone samples and expressed relative to the pre-supplementation values (Fig. 3C and D). Serum from subjects who ingested PSPC protected cells from the exercise-induced susceptibility to killing by rwt virus, as indicated by the significant post-exercise differences in cell viability between the two groups. This result was obtained when cells were incubated with serum for 0 (Fig. 3C) and 4 h (Fig. 3D) prior to virus infection. Furthermore, pre-incubating cells for 4 h with serum from PSPC-supplemented individuals also provided protection from virus infection following the 14-h recovery period. These data provide evidence that the serum of individuals ingesting PSPC contains antiviral properties that allow cells to resist virus infections following strenuous exercise.

Effect of polyphenol soy protein complex supplementation on virus replication

To further analyze the antiviral effects of PSPC-supplemented serum, the impact of serum on virus replication was measured. For these experiments, cells were infected with an rwt virus strain engineered to express green fluorescent protein (rwt-GFP) during its replication cycle (Whitlow *et al.*, 2006). At 6 h post-infection, a lower percentage of cells expressed GFP when incubated with serum obtained from PSPC versus placebo subjects immediately

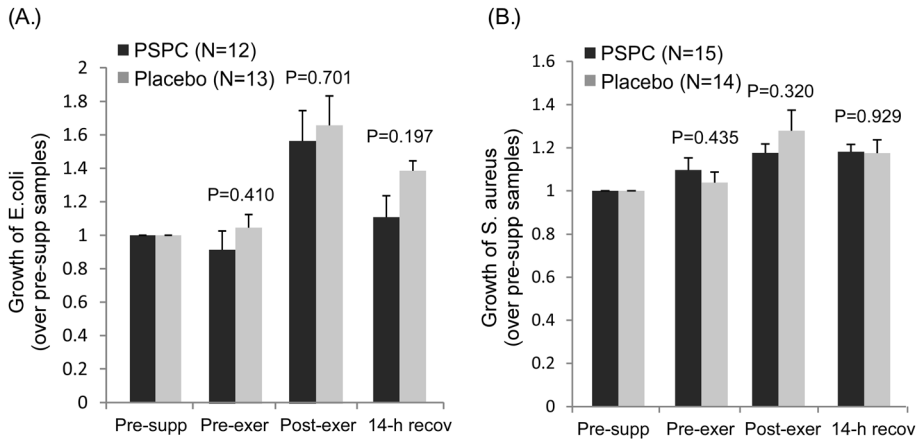

Figure 2. Growth of bacteria in the presence of serum from polyphenol soy protein complex (PSPC)-supplemented versus placebo-supplemented subjects. *Escherichia coli* (A) or *Staphylococcus aureus* (B) was incubated with PSPC or placebo serum for 24 h. Growth of bacteria was determined by measuring absorbance readings at 600 nm. All readings were normalized to the pre-supplementation values.

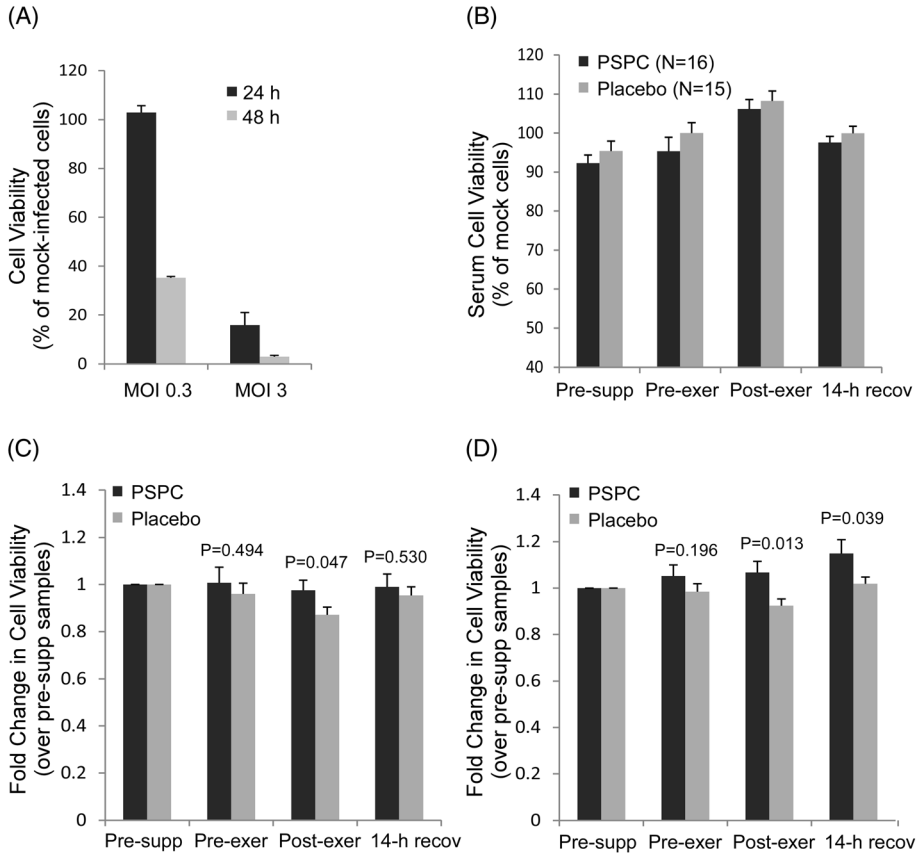

Figure 3. Antiviral activity in the serum from test subjects. HeLa cells were infected with serum from polyphenol soy protein complex (PSPC)-supplemented or placebo-supplemented subjects for different times and infected with rwt virus. Cell viability was measured by MTT assay. Controls included cells infected with virus alone or incubated with serum alone. All data were normalized to mock-infected cells. (A) Susceptibility of HeLa cells following infection with rwt virus at MOIs of 0.3 or 3 pfu/cell for 24 or 48 h. (B) Impact of serum alone on viability of HeLa cells (24 h). (C and D) Cells were incubated with serum from subjects for 0 (C) or 4 h (D), followed by vesicular stomatitis virus infection (rwt virus) for 24 h. Data were normalized to mock-infected cells and expressed relative to the pre-supplementation values.

post-exercise ($P = 0.061$) and at the 14-h recovery time period ($P = 0.038$) (Fig. 4A). This difference was not observed by 12 h post-infection (Fig. 4B). Consistent with this result, at 12 h post-infection, the geometric mean fluorescence was lower in the cells exposed to serum from PSPC-supplemented individuals as compared with serum from placebo subjects following exercise (Fig. 4D). These results imply that there was delay in virus infection when cells were incubated with PSPC versus placebo serum, as indicated by the 6 h result in Fig. 4A. Although virus recovered its ability to infect cells by 12 h post-infection, the degree of replication within cells was significantly lower during that time, as indicated by the geometric mean fluorescence data in Fig. 4D. Overall, we can conclude that PSPC serum contains factors that interfere with the ability of virus to effectively infect and replicate within cells. The effect of PSPC serum on virus replication is also consistent with the lowered impact of the virus on cell death, as shown in Fig. 3.

DISCUSSION

Numerous epidemiological and observational studies have suggested a link between strenuous exercise and increased susceptibility to virus infections in humans (Martin *et al.*, 2009; Nieman, 1994). Controlled animal studies have also supported this hypothesis. For example, exercise stress has been shown to be associated with an increased incidence and severity of HSV-1 and influenza respiratory infections (Brown *et al.*, 2007; Murphy *et al.*, 2008). Although various nutritional strategies have been investigated as a countermeasure to immune dysfunction during prolonged bouts of intense exercise, there is limited evidence that any of these strategies provide protection against susceptibility to infections. The results of our *ex vivo* studies indicate a protective effect of a PSPC on virus infectivity during a time when athletes are transiently immune-suppressed and susceptible to virus infections. Furthermore, the fact that serum from subjects ingesting PSPC significantly protects cells from the cytopathic effects of virus infection suggests that ingesting PSPC provides the body with soluble factors that have the potential to act systemically and target infections at different anatomical locations following strenuous exercise.

The mechanism by which PSPC and other flavonoids provide protection from virus infections remains to be determined. Cell culture studies have shown that the polyphenol, quercetin, inhibits the replication of several respiratory viruses, including influenza virus, adenoviruses and rhinoviruses, by targeting an early step in the virus infectious cycle (Cushnie and Lamb, 2005; Kim *et al.*, 2010; Selway, 1986). Consistent with this observation, we found that pre-incubation of cells with serum from PSPC-supplemented individuals for 4 h provided significant and sustained protection against killing by VSV, whereas the addition of serum at 0 h following viral infection did not result in sustained antiviral

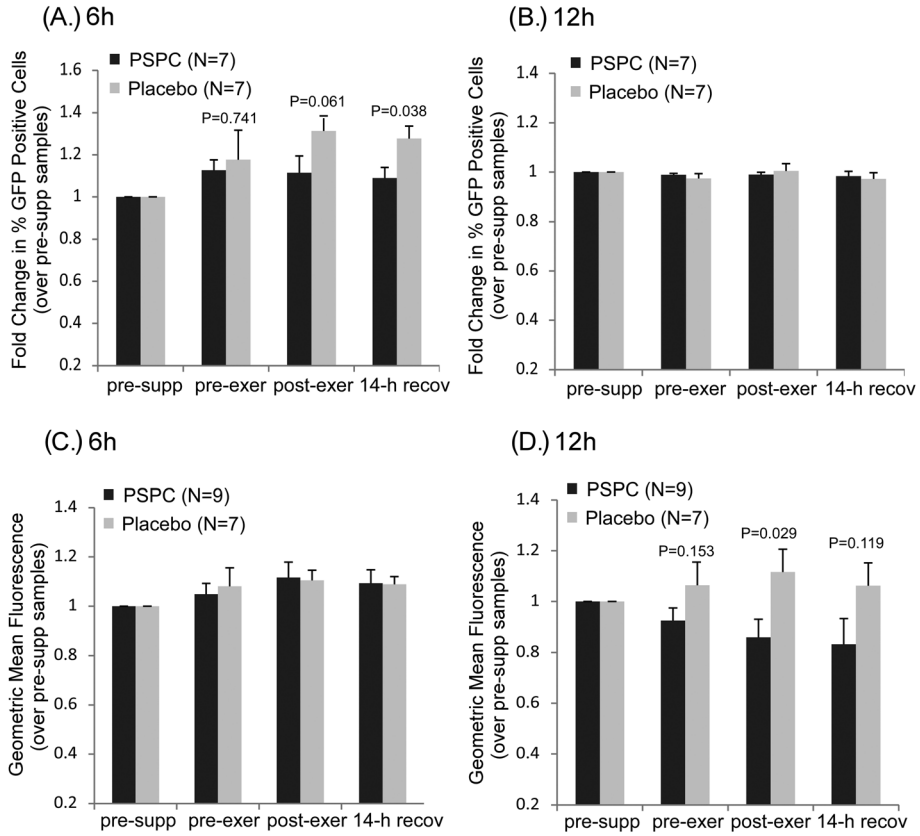

Figure 4. Replication of vesicular stomatitis virus in HeLa cells in the presence of serum from polyphenol soy protein complex (PSPC)-supplemented or placebo-supplemented subjects. HeLa cells were incubated with serum from PSPC or placebo subjects for 4 h and infected with rwt-GFP virus. At 6 or 12 h post-infection, GFP expression was measured by flow cytometry. Data were expressed as the percentage of cells expressing GFP at 6 (A) or 12 h (B) and the geometric mean fluorescence at 6 (C) and 12 h (D). All results were normalized to mock samples and expressed relative to the pre-supplementation values.

activity during the recovery period. This result suggests that PSPC in serum must induce antiviral activity prior to virus infection of cells in order to target and inhibit the early steps of the virus replication cycle. Proposed mechanisms for this suppression include blockage of virus binding to the host cell plasma membrane, alteration of essential viral proteins and induction of cellular antiviral responses targeting specific steps in the viral replication cycle.

Studies with influenza virus and hepatitis C virus (HCV) have attributed the antiviral effect of EGCG, a catechin found in green tea, to interference of viral penetration into cells due to damage of the virus particle (Calland *et al.*, 2012; Kim *et al.*, 2013). Defective internalization may be due to alteration of viral glycoproteins or physical damage to the viral particles, possibly the viral membrane. Similar to influenza virus and HCV, VSV is an enveloped virus that may also be susceptible to the damaging effects of EGCG. The cell surface receptors for VSV have not been identified, but binding of VSV to cells is thought to be unspecific and involving negative charges on the plasma membrane (Carneiro *et al.*, 2002). It is possible that EGCG or other factors in the serum of subjects ingesting PSPC affect the structure of the plasma membrane or the viral glycoprotein. The heat-inactivation of serum in our studies eliminated the non-specific effects of human complement at the viral envelope. Therefore, PSPC supplementation may provide additional factors that interfere with virus binding at the cell surface. In addition, we cannot exclude the possibility that alternative mechanisms also play a role in the antiviral activity of PSPC supplementation such as effects at the cellular level, including the inactivation of viral RNA polymerase or viral replication enzymes, binding of viral nucleic acid or viral capsid proteins, and suppression of virulence enzymes. PSPC contains blueberry extract, a rich source of anthocyanins and other polyphenols, that may induce antiviral effects by mechanisms that are distinct from those exerted by EGCG. Studies have suggested that in addition to affecting virus attachment to host cells, anthocyanins may inhibit the infectivity of influenza virus by inhibiting the cellular release of virus particles through binding to viral neuraminidase (Swaminathan *et al.*, 2013). Further studies will be carried out in order to investigate the exact mechanisms by which PSPC supplementation interferes with viral replication and killing of host cells.

The decreased resistance to virus infections during intense exercise has been shown to be partially due to the shift of balance towards the Th2 and away from the Th1 immune response (Martin *et al.*, 2009). Th1 inflammatory immune responses are critical for early antiviral activity including elevated surveillance by macrophages and dendritic cells, enhanced viral clearance, and memory responses. The protective effects of certain compounds following intense exercise have been attributed to the recovery of a Th1-type immune response leading to improved antiviral effector functions (Murphy *et al.*, 2004). For example, ingestion of oat beta-glucan enhanced macrophage function and improved the symptoms and survival of exercise-stressed mice following intranasal inoculation with HSV-1 (Davis *et al.*, 2004). In our PSPC

study, there were no observable differences in cytokine levels pre-exercise and post-exercise in placebo versus supplemented groups (Nieman *et al*., 2013). However, specific levels of Th1 cell-promoting factors such as IL-12, IL-2 and IFNγ were not measured in serum samples from subjects. Future studies will be aimed at determining the levels of Th1 cytokines in the serum of individuals following PSPC supplementation as further evidence of the antiviral properties of this compound.

Serum from PSPC-supplemented subjects in our study did not inhibit the growth of the gram negative bacteria, *E. coli*, or the gram positive bacteria, *S. aureus*. Consistent with our results, tea catechins have not been shown to impact the growth of *E. coli*, but instead, they demonstrate effectiveness by decreasing expression of virulence factors such as those responsible for biofilm formation and motility (Daglia, 2012). However, several *in vitro* studies have shown that EGCG inhibits the growth of *S. aureus* by binding to the positively charged lipids of the bacterial cell membrane (Steinmann *et al*., 2013). In addition, EGCG has been found to promote antibacterial activity by sensitizing *S. aureus* strains to the effects of specific antibiotics. It is possible that the levels of EGCG in the serum of PSPC-supplemented subjects were below the minimum inhibitory concentration for the *S. aureus* strain used in the study. EGCG is poorly absorbed from the human small intestine, but smaller phenolics following gut microbial degradation are translocated to the circulation during intensive exercise (Nieman *et al*., 2013). Little is known regarding the influence of these gut-derived phenolics on antibacterial activity. Alternatively, the effects of serum on bacteria may involve effects that were not measured in our study such as damage to the bacterial lipid layer or changes at the level of gene expression. Because serum from subjects ingesting PSPC exhibited antiviral properties, viruses may be more susceptible to the damaging effects of polyphenolic compounds at the plasma membrane than bacteria. In addition, the effect of polyphenols on cellular immune and stress response pathways may have more devastating downstream effects on intracellular pathogens. Further studies are necessary to definitively determine whether PSPC supplementation provides soluble factors that exert antibacterial effects.

Although the virus used in our study, VSV, is not a human pathogen, it serves as an effective model system for pathogenic human viruses due to its ability to replicate in cells from a variety of different species (Johannsdottir *et al*., 2009). Furthermore, it has been shown to infect cells from different organ systems, including respiratory epithelial cells and neurons. This study provides the framework for future studies aimed at determining whether the activity of PSPC in the serum of subjects exerts activity against pathogenic human viruses such as influenza virus, rhinovirus and HSV-1. Furthermore, examining the mechanisms by which polyphenols in PSPC interfere with the virus replication and exert antiviral activity enables the development of novel polyphenolic combinations with synergistic anti-microbial effects.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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