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MULTICENTRIC STUDY OF SEROPREVALENCE OF BORRELIA BURGDORFERI AND ANAPLASMA PHAGOCYTOPHILA IN HIGH-RISK GROUPS IN REGIONS OF CENTRAL AND SOUTHERN ITALY

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The aim of this study was to evaluate the seroprevalence of *B. burgdorferi* and *A. phagocytophila* in populations of workers from 4 Italian regions, known to be exposed to tick bites. A total of 712 serum samples collected were divided as follows: 387 samples were obtained from workers at risk for tick bites and 325 from individuals that were not considered to be at risk of ticks bites and served as the control group. Antibodies against *B. burgdorferi* were found in 29 (7.5%) of the 387 risk workers and in 4 (1.2%) of the 325 control group. Antibodies reactive with the HGE agent were found in 22 (5.7%) of the 387 risk workers and in 3 (0.9%) of the 325 control group. Antibodies to both *B. burgdorferi* and *A. phagocytophila* were found in 1.6% of the forestry workers confirming the possibility of coinfection or concurrent infection. The present finding show significant differences between seroprevalence of the risk workers and that of the people with no risk for tick exposure.

Ixodes ricinus, the most frequent and widespread tick species in Europe, may transmit various diseases such as *Lyme borreliosis* and human granulocytic ehrlichiosis (HGE) (1).

Lyme borreliosis, caused by the spirochete *Borrelia burgdorferi* s.l., is considered the most common tick-borne disease in Europe (2). In some areas of Northern Italy, *Lyme borreliosis* is endemic, while in other regions of the country, only sporadic cases have been reported (3). The clinical diagnosis of borreliosis is based on the typical symptoms observed as well as results from serological tests. Erythema chronicum migrans (ECM) is the typical early clinical symptom; later the disease presents arthritis, radiculoneuritis, cranial neuropathy, lymphocytic meningitis. In clinical practice indirect laboratory methods are widely used to support the diagnosis of borrelial infection.

HGE is caused by *Anaplasma phagocytophila*. In Europe, the first case of HGE was reported in Slovenia in 1997 (4), while in Italy two confirmed cases were reported in 2003 in the region of Friuli-Venezia-Giulia which are close to the Slovenian borders (5). HGE is an acute, febrile illness characterized by headache, myalgia, arthralgia, malaise, thrombocytopenia, leukopenia, and elevated levels of hepatic transaminases. The clinical diagnosis of HGE is often difficult because clinical manifestations and laboratory findings are non-specific (6). Tests to confirm the diagnosis during the acute phase include microscopic detection of morulae in granulocytes, culture of *A. phagocytophila*, and polymerase chain reaction. Confirmation by IFA is the most common diagnostic technique.

Key words: Anaplasma phagocytophila, Borrelia burgdorferi, risk workers

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At present, there is no single "gold standard" for the diagnosis of HGE.

Tick-borne zoonoses are known to be occupational diseases of particular working environments, mainly in the area of forestry and agricultural. The aim of this study was to evaluate the seroprevalence of *B. burgdorferi* and *A. phagocytophila* in populations of workers from 4 Italian regions, known to be exposed to tick bites.

MATERIALS AND METHODS

Study population

A total of 712 serum samples were collected from 4 Italian regions; Abruzzo and Latium of Central Italy, and Campania and Sicilia, from Southern Italy. The numbers of samples collected were divided as follows: 387 samples were obtained from workers at risk for tick bites and 325 from individuals that were not considered to be at risk of tick bites and served as the control group. The group of risk workers was further subdivided into 2 groups: 205 samples of gardeners and farmers defined at *low risk* and 182 samples of forestry workers defined at *high risk*.

A questionnaire was completed for each subject, describing the area of work, kind of job, exposure to ticks and Lyme borreliosis or HGE compatible symptoms.

Serological tests

The presence of IgG antibodies to *B. burgdorferi* was investigated in the collected samples using a commercially available enzyme-linked immunosorbent assay (ELISA) (MarDx, Diagnostic Inc., San Diego, CA) according to the manufacturer's instructions. Sera showing Lyme index values (LIV) ≥1.2 were considered positive, as recommended by the manufacturer.

In addition, the use of commercially available indirect immunofluorescence antibody (IFA) test kit (MRL Diagnostics, USA) was used to examine samples for anti-HGE IgG. This assay utilizes HL-60 cells infected with HGE-1 human isolate. Briefly, 25 ml aliquots of each serum sample, as well of IgG-positive and IgG-negative controls, were diluted in PBS and placed on a slide-well plate, in contact with the substrate. Slides were incubated in a humid chamber for 30 minutes at 37°C. After washing, 25 ml of IgG-conjugate were added to each slide-well and incubation was continued for further for 30 min. Wells were washed, dried, mounted and examined using fluorescence microscopy. Positive and negative-controls were included in each run. Sera showing titres ≥ 1:64 were considered positive.

Statistics

Statistical analysis was performed by Fisher and χ^2 tests.

RESULTS

Antibodies against *B. burgdorferi* were found in 29 (7.5%) of the 387 risk workers and in 4 (1.2%) of the 325 control group. Of the 29 seropositive risk workers, 23 (12.6%) were among the high risk group and 6 (2.9%) among the low risk group (Tab. I). The highest percentage of positive was noted in risk groups from Southern Italy 13.7% (Tab. I).

Antibodies reactive with the HGE agent were found in 22 (5.7%) of the 387 risk workers and in 3 (0.9%) of the 325 control group. Of the 22 seropositive risk workers, 19 (10.4%) were among the high risk group and 3 (1.5%) among the low risk group (Tab. II). The highest percentage of positive was noted in risk groups from Southern Italy 11.4% (Tab. II).

Three (1.6%) forestry workers were seropositive for both *B. burgdorferi* and *A. phagocytophila*.

Among data from questionnaires, 65 (35.7%) forestry workers reported a history of tick-bite, while none of the low risk group and control group reported it.

DISCUSSION

In Italy, the cases of Lyme borreliosis have been reported from many Italian regions (7), while until now only two cases of HGE have been reported from Friuli-Venezia-Giulia (5) and the detection of antibodies to *A. phagocytophila* has been reported from Veneto, Abruzzo and Latium (8-11). The present findings show significant differences between seroprevalence in high risk workers compared to subjects with low risk of tick exposure. In fact, seropositive results were found more frequently in the risk workers than in the control group in both the IgG for *B. burgdorferi* (7.5% vs. 1.2%, p=0.0002) and the IgG for *A.*

Tab. I. *Frequency of seropositive to B.burgdorferi in risk groups and control group from Central and Southern Italy.*

Group	Central Italy (Latium/Abruzzo)		Southern Italy (Sicilia/Campania)		Total positive	
	No.[c]	IgG+ (%)	No.[c]	IgG+ (%)	No.[c]	IgG+ (%)
Risk group						
High risk[a]	37	2 (5.4)	145	21 (14.5)	182	23 (12.6)
Low risk[b]	175	3 (1.7)	30	3 (10.0)	205	6 (2.9)
Total	212	**5 (2.4)**	175	**24 (13.7)**	387	**29 (7.5)**
Control group	180	**1 (0.5)**	145	**3 (2.1)**	325	**4 (1.2)**

[a]*High risk: Forestry workers*
[b]*Low risk: Gardeners/ Farmers*
[c]*No.: Number of serum samples tested*

Tab. II *Frequency of seropositive to HGE in risk groups and control group from Central and Southern Italy.*

Group	Central Italy (Latium/Abruzzo)		Southern Italy (Sicilia/Campania)		Total positive	
	No.[c]	IgG+ (%)	No.[c]	IgG+ (%)	No.[c]	IgG+ (%)
Risk group						
High risk[a]	37	1 (2.7)	145	18 (12.4)	182	19 (10.4)
Low risk[b]	175	1 (0.6)	30	2 (6.7)	205	3 (1.5)
Total	212	**2 (0.9)**	175	**20 (11.4)**	387	**22 (5.7)**
Control group	180	**1 (0.5)**	145	**2 (1.4)**	325	**3 (0.9)**

[a]*High risk: Forestry workers*
[b]*Low risk: Gardeners/ Farmers*
[c]*No.: Number of serum samples tested.*

phagocytophila (5.7% vs. 0.9% p=0.001) (Tab. I and Tab. II). The higher prevalence observed in the risk group is an indication of the fact that groups that work outdoors are more likely to get exposed to the infection. This supports the occupational character proposed for tick-borne zoonoses among risk workers This multicentric study reports a seroprevalence of *B. burgdorferi* in risk workers, 7.5%, and in control group, 5.7%, higher than that of HGE, 1.2% and 0.9% respectively, suggesting a higher diffusion of *B. burgdorferi* in studied regions. Moreover in our study there was a significantly higher prevalence of *B.burgdorferi* (p=0.0006) and *A. phagocytophila* (p=0.0003) in *high risk* group than in *low risk* group, confirming that forestry workers are the populations with a higher individual risk for tick-borne diseases (Tab. I and Tab. II). Moreover in this work statistical analysis has shown significant differences between the prevalence of the risk group of Southern Italy and that of the risk group of Central Italy in both *B. burgdorferi* (13.7% vs. 2.4%, p=0.00005) and HGE (11.4% vs. 0.9%, p=0.00002), suggesting a higher diffusion of *B. burgdorferi* and HGE in Southern Italy (Tab. I and Tab. II).

In Europe serological studies conducted on

high risk groups show a great variation of positive prevalence within and between species, 5.7 to 71% for *B. burgdorferi* (12) and 3.8 to 25 % for *A. phagocytophila* (6). Eleven of the seropositive for *B. burgdorferi* and *A. phagocytophila* remembered one or more tick-bites and none showed clinical manifestations compatible with Lyme borreliosis or HGE. The seropositive subjects did not show clinical signs of borreliosis or HGE, suggesting the possibility of asymptomatic infections. This is in agreement with other European authors (13-15) who believe that asymptomatic infections occur in individuals frequently exposed to ticks. Antibodies to both *B. burgdorferi* and *A. phagocytophila* were found in three forestry workers, confirming the possibility of coinfection or concurrent infection. This possibility has been documented in other Italian and European studies, suggesting that the coinfection may be a common occurrence in individuals who have high probability of tick exposure and may have had multiple tick bites (6, 16-19). Moreover, PCR studies in *I. ricinus* reveal coinfection of ticks with *B. burgdorferi* and *A. phagocytophila*, confirming the possible transmission of these two agents in a host (20-22). While cases of Lyme borreliosis have been reported from Campania and Sicilia showing the circulation of *I. ricinus* (7), to our knowledge this multicentric study documents for the first time the presence of seropositivity to HGE in these regions.

In conclusion, our study indicates that Lyme borreliosis and HGE are present in central and southern regions of Italy and that forestry workers from from these regions can be considered at high risk of infection with *B. burgdorferi* and HGE. Therefore, HGE should be taken into account in the differential diagnosis of febrile illnesses. Further prospective studies are indicated to evaluate the clinical importance of Lyme borreliosis and HGE in Italy.

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N-ACETYLCYSTEINE SYNERGIZES WITH OSELTAMIVIR IN PROTECTING MICE FROM LETHAL INFLUENZA INFECTION

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Many studies have shown that oxidative stress is important in the pathogenesis of pulmonary damage during influenza virus infections. Antioxidant molecules are therefore potentially useful against viral infection. Our previous studies show that N-acetylcysteine (NAC) has a protective effect in a model of lethal influenza infection in mice. NAC administration significantly decreased the mortality in infected mice. Further studies have demonstrated that NAC enhanced survival in combination with the antiviral agent ribavirin. In the present study, we report the effect of combined treatment with NAC and Oseltamivir, clinically used in the treatment and prevention of influenza virus infection, in a murine model of lethal influenza infection. NAC was given as a single daily dose of 1000 mg/Kg starting from 4 h before infection and until day 4 after infection; Oseltamivir was given twice daily at dose of 1 mg/Kg/die for 5 days, starting from 4 h before infection. End-point evaluation was 21-days' survival. NAC alone was slightly effective (20%), since a suboptimal treatment was used. Survival increased to 60% with Oseltamivir and to 100% with Oseltamivir and NAC used in combination. Since NAC alone does not show any antiviral action, the present findings suggest that antioxidant therapy increase survival by an improvement in host defense mechanisms, and/or by a direct antioxidant effect against oxidative stress associated with viral infection. Our studies demonstrate the effectiveness of combining agents acting through different mechanisms, such as antiviral drugs oseltamivir and the antioxidant NAC, indicating a possible advantage of combining the two treatments.

Various mediators contribute to the pathogenesis of pulmonary inflammation induced by infectious agents. In particular, cytokines, chemokines and reactive oxygen species (ROS) have been implicated. Cytokines and chemokines, which are both produced as part of the host immune response to bacteria (1-2), contribute to the pathogenesis of tissue damage (3-4). ROS contributes to the antibacterial response, even if overproduction can result in oxidative stress that amplifies the inflammatory response.

Many studies have shown that oxidative stress is important in the pathogenesis of pulmonary damage during infections, in organ failure and in acute respiratory distress syndrome (ARDS) associated with bacterial sepsis. Antioxidant molecules are therefore potentially useful against both viral infection and infection-associated symptoms.

Oxidative stress and its role in the pathogenesis of various infections has been documented in experimental models both *in vitro* and *in vivo* using a variety of pathogens: e.g. *Chlamydia pneumoniae* (5), *Helicobacter pylori* (6), different Gram-

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Table I. *Effects of Oseltamivir and NAC on 21-days' survival.*

Treatment[a]	Dosage (mg/Kg/die)	N° of survivors/total[b]	Survival rate (%)
Oseltamivir	1.0	6/10	60
Vehicle (control)	0	0/10	0
NAC	1000	2/10	20
Vehicle (control)	0	1/10	10
Oseltamivir + NAC	1.0 plus 1000	10/10	100
Vehicle (control)	0	0/10	0

[a] *Treatment, per os, started 4 h before infection and for the subsequent 4 days*
[b] *Mice were observed daily for 21 days for survival*

Table II. *Effects of Oseltamivir and NAC on the lethality of influenza infection in mice.*

Treatment[a]	MST (N° of days)[b]
Oseltamivir (n=10)	21.7
NAC (n=10)	8.8
Oseltamivir + NAC (n=10)	> 21 *
Vehicle (n=30)	8.7

[a] *Treatment, per os, started 4 h before infection and for the subsequent 4 days*
[b] *Results are reported as mean survival time (MST) in days, estimated by the Kaplan-Meier method*
* *$p<0.05$ compared to Oseltamivir alone by Wilcoxon test*

negative bacteria (7), *Streptococcus pneumoniae* in an animal model of pneumococcal meningitis (8-9), HIV (10-12) and Hepatitis C virus (HCV) (13-16). ROS, through the induction of several inflammatory cytokines, have also been implicated in the pathogenesis of SARS (17).

The possible involvement of oxidative mechanisms in the pathogenesis of influenza virus infection has also been investigated. Intranasal instillation of influenza virus H1N1 in mice resulted in a significant decrease in the pulmonary concentrations of catalase, reduced glutathione and superoxide dismutase. Furthermore, the H5N1 virus (A/Hong Kong/483/97), when compared with human influenza virus subtype H1N1, is a more potent inducer of pro-inflammatory cytokines (e.g. tumor necrosis factor-a) and chemokines (e.g. IP-10) from primary human macrophages *in vitro*. This characteristic may contribute to the unusual severity of human H5N1 disease (18-19).

Previous studies have shown that N-acetylcysteine (NAC), a thiol antioxidant and

precursor of GSH synthesis, has a protective effect in a model of lethal influenza infection in mice. NAC administration significantly decreases the mortality in infected mice, presumably by limiting tissue damage (20). Further studies, using the same *in vivo* model, have demonstrated that NAC, at doses that do not improve survival of mice when given alone, enhances survival in combination with the antiviral agent ribavirin (21).

In the present study we report the effect of combined treatment with NAC and Oseltamivir, a neuroaminidase inhibitor clinically used in the treatment and prevention of influenza virus infection, in a murine model of lethal influenza infection.

MATERIALS AND METHODS

BALB/c mice (18-20 g), 10 per treatment group, were anaesthetized with ether/chloroform and infected intranasally with 2-3 × LD_{50} of an influenza virus strain adapted in mice (A/PR8/H1N1). Animals were treated as follows:

1) Oseltamivir alone, 1 mg/Kg/die, per os, twice daily for 5 days, starting from 4 h before infection and for the subsequent 4 days;
2) NAC alone, 1000 mg/Kg/die, per os, starting from 4 h before infection and for the subsequent 4 days;
3) Combined treatment with Oseltamivir and NAC as described above.

Each experimental group was associated to a respective control group, receiving only the vehicle. Mice were observed daily for 21 days for survival. Mean survival time (MST) was calculated as previously described (22).

RESULTS

The effect of treatment of influenza-infected mice with Oseltamivir and NAC used alone and in combination on 21-days' survival is shown in Table I. A control group, receiving only the vehicle, was used for each treatment.

Treatment with Oseltamivir increased the survival of infected mice from 0 to 60%. NAC alone was slightly effective (20%), since a suboptimal treatment was used, but it significantly increased survival in combination with Oseltamivir (average, 100% vs. vehicle-treated control mice).

Table II shows the estimated mean duration of survival. All control animals died 7 to 11 days after infection with influenza virus. Although NAC used alone did not increase survival, when used in combination with Oseltamivir it improved antiviral efficacy and enhanced survival time.

The difference between treatment with Oseltamivir used alone and in combination with NAC is statistically significant.

DISCUSSION

The present study confirms our previous report of a positive effect of NAC administration in combination with an antiviral drug in a murine model of influenza infection. In this report we used a neuraminidase inhibitor, Oseltamivir, clinically used in the treatment and prevention of influenza virus infection.

Since our previous paper reported that NAC alone does not show any antiviral action (20), the present finding could be explained by an improvement in host defense mechanisms, and/or by a direct antioxidant effect against oxidative stress associated with viral infection.

Since NAC is a precursor of GSH synthesis, the data indicate that GSH could play a key role in the host response to infections. Many authors have demonstrated that GSH prevents pulmonary damage induced by sepsis or bacterial endotoxins, suggesting a protective role on the pathogenesis of pulmonary damage (23-27). This is also in agreement with previous reports indicating that influenza infection decreased pulmonary antioxidants, including catalase, glutathione and superoxide dismutase (28), while increasing the activity of the superoxide-generating enzyme xanthine oxidase (XO) (29). Therefore, various antioxidant substances might prevent lung injury in a mouse model of influenza infection (20, 30).

Glutathione and NAC might also increase the host defense against influenza infection. Our studies demonstrate no direct antiviral effect of NAC on influenza infection *in vitro*, while other authors (31) report that GSH has a dose-dependent anti-influenza effect in cultured cells. Protection was also observed with an *in vivo* mouse model. Moreover, GSH prevented apoptosis of infected cells via inhibition of viral induced caspase activation.

On the contrary, oxidative stress, induced by exposure to diesel exhaust (DE), enhanced the

susceptibility to influenza virus infection in all cell models and the addition of the antioxidant GSH reversed the effects of DE on influenza infections (32).

Studies on virus (HIV)-infected patients have shown that GSH is important for T cell-mediated immunity; in fact, the treatment with anti-oxidants caused a significant increase in all immunological functions, including an almost complete restoration of natural killer cell activity (33). In a model of peritoneal sepsis in mice, GSH depletion caused increased infection and ARDS, while NAC augmented antibacterial functions in the peritoneum, decreased infection and improved survival (34). Overproduction of ROS can be toxic to cells of the immune system and suppress the immune response. For instance, phagocyte-generated ROS suppress natural killer (NK) T cells (35), can impair phagocytic functions and bactericidal activity of macrophages through *de novo* synthesis of actin or actin oxidation in patients with inflammatory lung diseases (36).

A clinical study shows that NAC administration to influenza-infected patients reduces both local and systemic symptoms and increases cell-mediated immunity (37).

In conclusion, we are constantly exposed to ROS generated from endogenous and some exogenous sources (e.g. viral infection). These ROS react with biological molecules causing structural and functional damage.

Antioxidants limit oxidative damage to biological molecules by various mechanisms and contribute to antioxidant defense systems in humans, and may help to protect against degenerative diseases. Our studies demonstrate the effectiveness of combining agents acting through different mechanisms, such as antiviral drugs including ribavirin and oseltamivir and the antioxidant NAC, indicating a possible advantage of combining the two treatments. Further experiments and clinical studies are necessary to confirm this hypothesis.

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INHIBITORY ACTIVITY OF CRANBERRY EXTRACT ON THE BACTERIAL ADHESIVENESS IN THE URINE OF WOMEN: AN EX-VIVO STUDY

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Strains of uropathogenic *E. coli* are responsible for approximately 90% of community-acquired, uncomplicated cystitis, and fimbriae represent the adhesive factors enabling *E. coli* to be anchored to uroepithelial cells in the first step of the infectious process. Recently, a few studies have shown that a correlation between the consumption of cranberry (*Vaccinium macrocarpon*) and prevention of UTI is related to the ability of proanthocyanidins to reduce the bacterial adhesion to uroepithelial cells. In this study we evaluate the inhibitory activity of urine of healthy women treated with tablets containing cranberry extract on the adhesiveness of *E. coli* to uroepithelial human cells. Two groups of 12 female volunteers each, aged between 18 and 65 years, were enrolled, one group with negative history and one group with positive history of recurrent cystitis. Subjects were treated with the active product or placebo in a random, cross-over, double-blinded sequence for one week in each of the two treatment sequences. Urine samples were collected at the beginning and the end of each study period. Tests of bacterial adhesiveness were performed with two strains of *E. coli* (ATCC 25922 and ATCC 35218) on HT1376 human bladder carcinoma cells. Significant reductions of bacterial adhesiveness were observed in women who received cranberry extract (-50.9%; p<0.0001), regardless of their medical history and the treatment period in the cross-over sequence. No changes were observed with placebo (-0.29%; n.s.). This *ex-vivo* study showed that the assumption of cranberry extract in suitable amounts can have an anti-adhesive activity on uropathogenic *E. coli*.

Urinary tract infections (UTI) are very common diseases, both in hospitals and the community (1-2), with *Escherichia coli* as the primary causative agent (3). Strains of uropathogenic *E. coli* (UPEC) are responsible for approximately 90% of community-acquired, uncomplicated cystitis (4). These infections are much more common among women, primarily because of a shorter urethra than in men. Twenty-five to 50% of women experience at least one episode of UTI in life and run into more or less frequent recurrences.

Fimbriae represent the adhesive factors enabling *E. coli* to be anchored to uroepithelial cells in the first step of the infectious process. Two types of fimbiae are present on *E. coli*: mannose-sensitive type 1 fimbriae binding to mannose-containing glycoproteins (5) and mannose-resistant P fimbriae binding to α-D-Gal(1.4)-β-D-Gal, a galactose disaccharide. Type 1 fimbriae are present on all UPEC, while P fimbriae are found less frequently.

Key words: anti-adhesiveness activity, cranberry extract, E. coli, recurrent cystitis

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However, both mediate the adhesion of *E. coli* to uroepithelial cells (6-7) and are responsible for the persistence of *E. coli* in the urinary tract and eventually the recurrence of the infectious process.

As multiple resistance to antibiotics has become a critical clinical issue(8), also in UTI, the development of new methods for the prevention and treatment of UTI has become of key importance. Recently, a few studies have shown a correlation between the consumption of cranberry (*Vaccinium macrocarpon*) and prevention of UTI (9). Cranberries are composed of a complex mixture of organic acids, vitamin C, flavonoids and anthocyanidins, among others (10). The anthocyanidins and proanthocyanidins are polyphenols with high antioxidant and radical scavenging activity (11), functioning as a natural plant defence system against microbes. The anti-infective efficacy of cranberry has been shown to be related to the ability of proanthocyanidins to reduce the bacterial adhesion to uroepithelial cells (12-14).

The present study was performed to evaluate the inhibitory activity of the urine of healthy women treated with a dietary supplement based on tablets containing cranberry extract and vitamin C on the adhesiveness of *E. coli* to human uroepithelial cells.

MATERIALS AND METHODS

This was a randomized, double-blind, cross-over, placebo-controlled, study with stratified randomization. The randomization list was prepared by an appointed statistician, by means of a computerized program implemented in SAS environment. The study was approved by the Institutional Review Board / Ethics Committee of the S.S. Salvatore Hospital of Paternò, Catania (Italy), and was conducted in the gynecology department. Twenty-five volunteers were randomized but one subject did not complete the study because of the appearance of an exclusion criterion at the start of the second cross-over period. Twenty-four women aged between 18 and 65 years were evaluated for the study: 12 healthy female volunteers with no history of recurrent cystitis and 12 women with history of recurrent cystitis (3-5 episodes of uncomplicated cystitis in the 12 months preceding the start of the study).

The sample size was calculated taking into account an average difference between treatments of 3.49 in the adhesion index (2.7 ± 2.44 in the cranberry group *vs* 6.19 ± 4.92 in the placebo group), by assuming a standard deviation of the difference of 3.68 and a two-tail significance level of 5%. The sample size of 12 subjects by sequence guarantees to the 2x2 crossover design a statistical power > 90% to detect the targeted difference between treatments and with 80% power to test the same difference within a single stratum.

All subjects signed an informed consent. The following criteria were a reason for excluding subjects from the study: antibiotic treatment in the two weeks preceding enrollment, suspected or actual current urinary tract infection (positive urine culture), treatment with vitamin K antagonists, history of kidney stones, pregnancy and, in general, all those conditions that might interfere with compliance of treatment and procedures. Fertile women started the first treatment immediately after the cyclic menses, in order to avoid having menses during one of the two study periods.

The subjects underwent 5 visits. At baseline (visit 1), medical history was collected to evaluate the inclusion/exclusion criteria, a physical examination was carried out and a sample of urine was collected for urine culture. During visit 2, the subjects were randomly assigned to one of two treatment sequences, another urine sample was collected for the evaluation of the adhesion index and they were instructed to take the study product every night for seven consecutive days. At visit 3, in the morning after the last administration, another urine sample was collected for the evaluation of the adhesion index after the first treatment period. After a wash-out period of one week, at visit 4 (baseline for the second treatment period) a urine sample was collected for urine culture (to exclude any current urinary tract infection) and the evaluation of the adhesiveness index, and the second treatment kit was delivered with instructions to take the study product every night for seven consecutive days. Visit 5 was the last study visit in the morning after the last administration during which a urine sample was collected for the evaluation of the adhesion index after the second treatment period.

The study product (Monurelle®, Zambon SPA, Bresso - Milano, Italy) was available in tablets containing 120 mg cranberry extract (including 36 mg proantocyanidines) and 60 mg ascorbic acid for oral assumption (once daily at night, for one week).

The dose of 36 mg proantocyanidines is in line with the declarations of the Agence Française de Sécurité Sanitaire des Produits de Santé (AFFSAPS) for the non-antibiotic prophylactic treatment of recurrent cystitis (15).

Blinding was permitted by the availability of kits specifically manufactured and labelled for this study which contained the active product or placebo which were identical in aspect and taste.

The *in vitro* bacterial adherence experiments were performed using an adaptation of the method of Di Martino et al. (16). Each urine sample was centrifuged at 3000 rpm

for 10 minutes, sterilized by filtration (0.45 μm) and stored at a temperature of -20°C. Adhesion tests were performed using HT1376 cells from human bladder carcinoma. Cells were cultivated in a 24-well plate at 37°C in Minimal Essential Medium (MEM) with 10% fetal bovine serum, 2 Mm glutamine and 100 μg/ml streptomycin. Before infection the cells were washed with PBS to remove the antibiotic present in the culture medium.

The examined strains were *E. coli* ATCC 25922, *E. coli* ATCC 35218. Both express P fimbriae and type 1 fimbriae. Bacteria were grown for 36 h in urine samples, added with 5% Luria Bartani broth. Bacteria were collected by centrifugation and resuspended in MEM at a concentration of 0.5 McFarland, approximately 10^8 CFU/ml. Thereafter, the bacterial suspensions were put in contact with the uroepithelial cells and incubated at 37°C for 3 h. After three washings with PBS, cells were fixed with methanol, stained with 10% Giemsa and examined under a microscope.

The adhesion index was determined as the average number of adherent bacteria per cell, resulting from the examination of 100 cells. Each test was repeated 3 times (16).

The means of the adhesion indices at the end of each cross-over period were analyzed by a mixed model of ANOVA for cross-over design. The means of the two treatments and the differences between test product and placebo were estimated by the fitted models with the 95% confidence limits.

RESULTS

The 24 subjects evaluated for the study were all Caucasian, aged between 20 and 57 years. Twelve had history of recurrent cystitis. Placebo did not exert any relevant effect in either group of volunteers, in any period of the cross-over sequence. In contrast, values of bacterial adhesiveness obtained from the urine of women taking the cranberry supplement showed a significant reduction in both groups of volunteers. The means (± SD) of the bacterial adhesion index were 2.89 ± 0.56 for the cranberry extract and 5.83 ± 0.96 for placebo in the overall study population, at the end of the treatment. Similar differences were observed within the two groups of volunteers: 3.02 ± 0.72 *vs* 5.85 ± 0.98 in subjects with no history of recurrent cystitis and 2.77 ± 0.31 *vs* 5.82 ± 0.97 in subjects with history of recurrent cystitis (active *vs* placebo, respectively) (Table I). The effect of the treatment cleaned by any other source of variation was highly significant ($p < 0.0001$) in both populations.

The least square means of bacterial adhesion indices by treatment and the difference between treatments is represented in Table II with 95% confidence limits.

The results, divided into the two groups of volunteers and by period of treatment, are shown in Fig.1 (subjects with no history of recurrent cystitis) and Fig. 2 (subjects with history of recurrent cystitis). Fig. 3 shows the effect of treatment.

By comparing the outcomes in the overall population by sequence, the index resulted 3.07 *vs* 5.73 in period 1, and 2.69 *vs* 5.92 in period 2, for active and placebo respectively. Almost the same difference was maintained after comparison of the treatments by sequences within the single strata: 3.32 *vs* 5.36 in period 1 and 2.60 *vs* 6.20 in period 2 for women with no history of recurrent cystitis; 2.78 *vs* 6.05 in period 1 and 2.76 *vs* 5.60 in period 2 for women with history of recurrent cystitis.

Table I. *Anti-adherence activity of cranberry extract vs placebo in female subjects with and without history of recurrent cystitis.*

	No history of recurrent cystitis		history of recurrent cystitis	
	cranberry extract	placebo	cranberry extract	placebo
Mean	3.02	5.85	2.77	5.82
SD	0.72	0.98	0.31	0.97
Median	2.83	5.62	2.70	5.55
Min - Max	2.33 – 4.97	4.60 – 7.83	2.43 – 3.58	4.67 - 7.90

Table II. *Means of adhesion indices and 95% confidence limits.*

	bacterial adhesion index	95% CONFIDENCE LIMITS		p-value T-test
		Lower limit	Upper limit	
cranberry extract	2.86	2.66	3.06	<.0001
placebo	5.81	5.61	6.00	
difference cranberry -placebo	-2.95	-3.23	-2.67	

The overall percentage reduction of bacterial adhesiveness was 50.9% with cranberry extract and 0.29% with placebo. In women without history of recurrent cystitis, the percentage reduction of bacterial adhesiveness between baseline and end-treatment was 47.5% and 52.3% in the two periods with active treatment. Also in women with history of recurrent cystitis there was a significant reduction of 50.8% and 54.0% in the two periods with active treatment.

No significant differences were seen between the two groups of volunteers treated with cranberry extract (52.4% and 49.5% reduction rates in women with or without recurrent cystitis, respectively; p = n.s.).

The analysis of variance did not show any significant effect of period (p= 0.4658), sequence (p= 0.3676) or stratum (p= 0.6981).

The treatments were well-tolerated with no adverse effects in either study group, during or after the treatment administration.

DISCUSSION

This *ex-vivo* study showed a significant effect of tablets containing cranberry extract + vitamin C in decreasing the bacterial adhesion of *E. coli* to human uroepithelial cells. The magnitude of effect was large and determined a reduction by approximately 50% of the adhesion index, while no effect was seen with placebo.

Traditional medicine has used cranberries for its antiseptic effect for generations (10). It is also widely used nowadays: in a survey of non-pharmacological remedies used during pregnancy, cranberry resulted amongst the most popular (reported by 43% of responders), and was used for UTI prevention (17). As reported in a Cochrane Database Systematic Review on the use of cranberry for the prevention of UTI (9), including trials up to January 2007, two well-conducted, controlled, clinical trials have shown an antibacterial effect in symptomatic UTIs in women, with significant reduction in the incidence of UTIs at twelve months (RR 0.61; 95% CI:0.40 to 0.91) compared with placebo or control. More recently, an open-label study not referenced in the Cochrane review confirmed prevention of UTI in women with a history of recurrent infections, during a follow-up of 12 weeks in twelve subjects and over 2 years in 8 subjects (18). In another study of 137 older women with ≥ 2 UTI in the year prior to enrolment, the treatment with 500 mg of cranberry extract or 100 mg of trimethoprim for 6 months showed no significant differences in the occurrence of UTI, though with more episodes in the cranberry group (19). Despite favorable data in the prevention of UTI, no real effect of cranberry has been demonstrated in patients with an already established UTI.

Recent studies have shown that cranberry extract inhibit bacterial adhesion to uroepithelial cells (12, 14, 20-21), which is the first step of the infectious process of UTI, by inhibiting the bacterial agglutination. Cranberry affects the adhesion forces within a few hours of exposure, only on fimbriated bacterial strains (22). Another possible mechanism

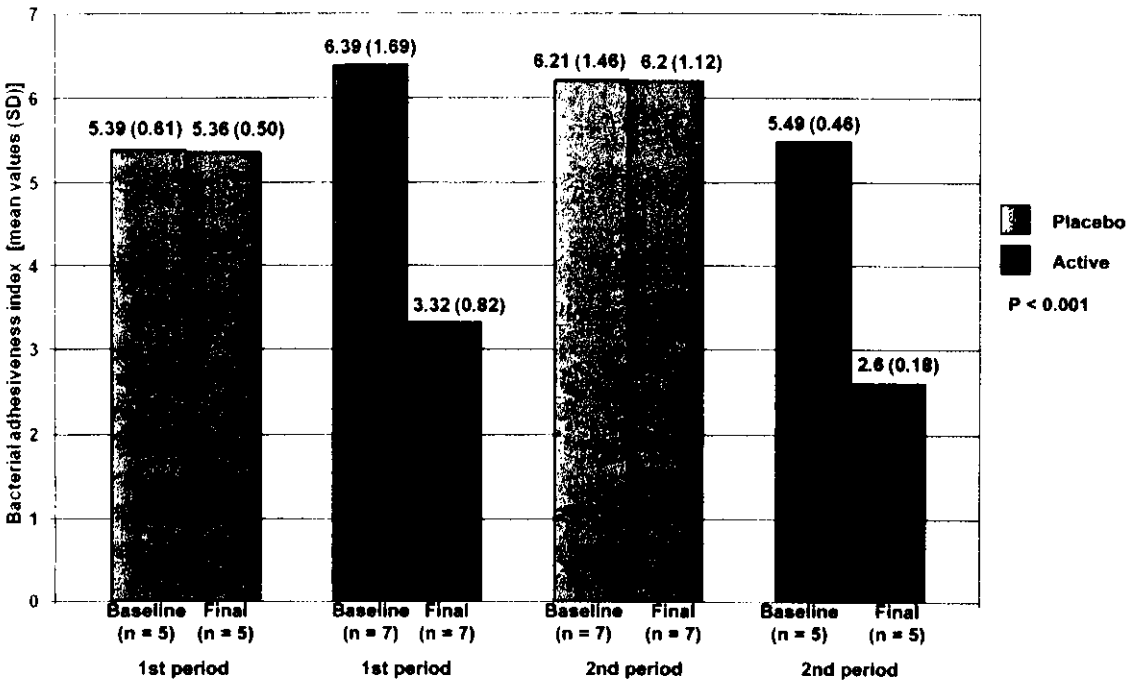

Fig. 1. *Female volunteers with no history of recurrent cystitis.*
(P< 0.001: level of significance of the treatment effect by ANOVA for cross-over design)

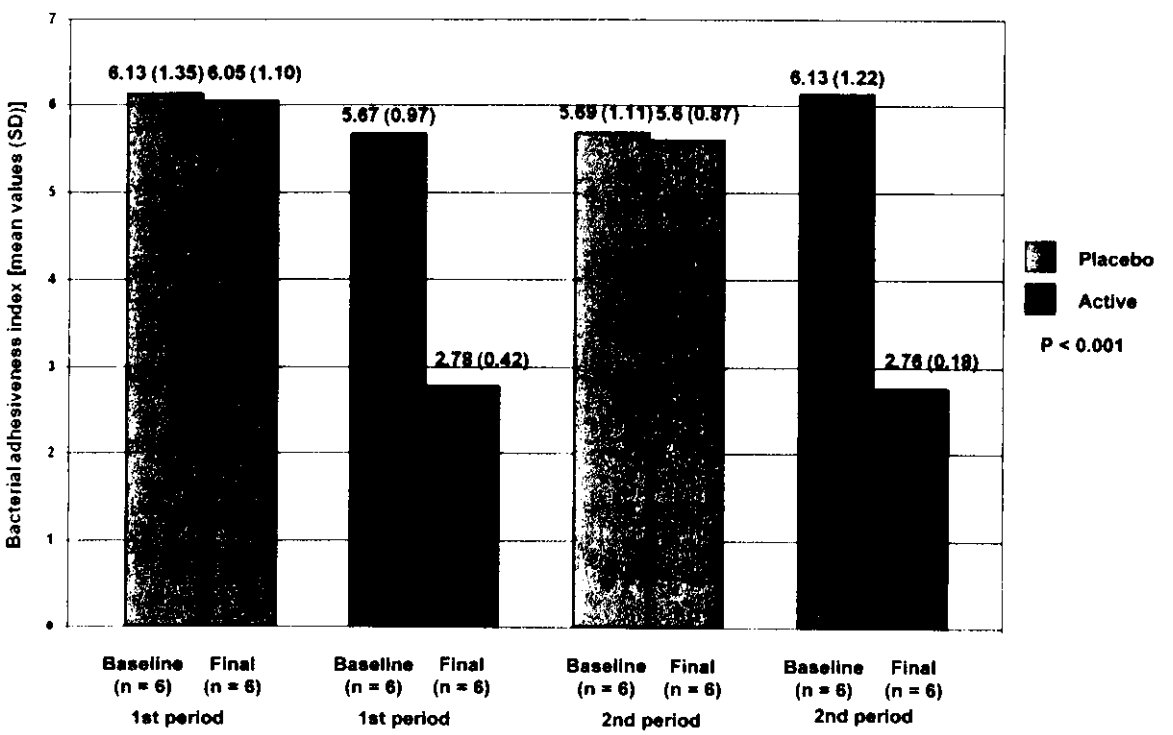

Fig. 2. *Female volunteers with history of recurrent cystitis.*
(P< 0.001: level of significance of the treatment effect by ANOVA for cross-over design)

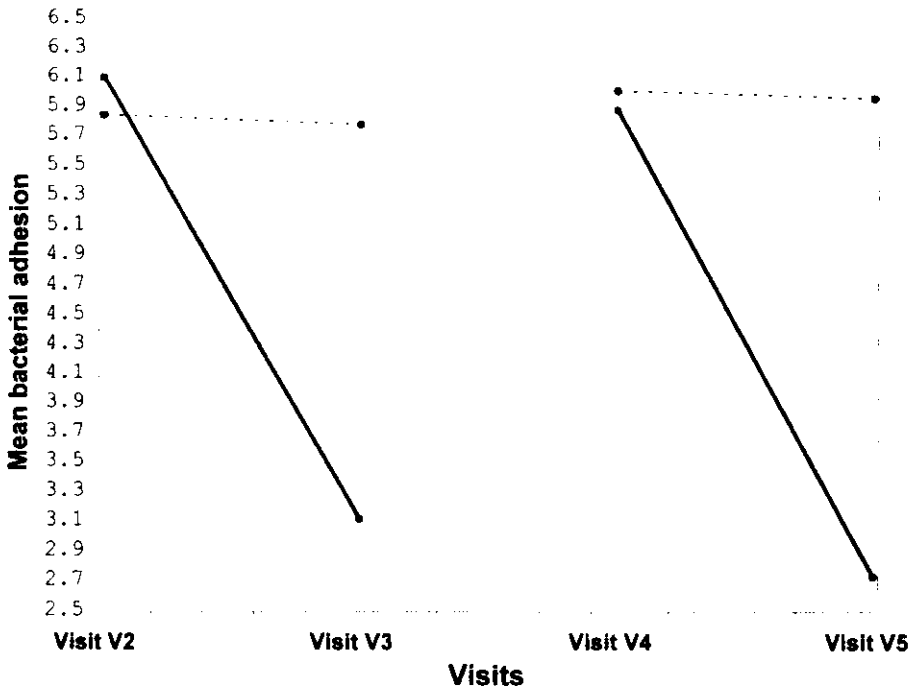

Fig. 3. *Representation of effect by treatment. Full line(•——•): Cranberry plus Vitamin C Dotted line (•------•): Placebo*

which may be active in the long term was identified in the modulation of the expression of bacterial fimbriae (23).

The potent anti-adhesive property of cranberries is regarded as the effective factor in the prevention of UTI. This is mediated by the anthocyanidin/ proanthocyanidin moieties which are thought to be the main active constituents in cranberry. In particular, A-type proanthocyanidin are active in cranberry, eliciting higher *in vitro* anti-adhesion activity than B-type, which is available in other food. This possibly explains the difference between various tannin-rich foods in the anti-adhesion activity (24). The ability of cranberry to prevent infections of the urinary tract was also attributed to fructose (25). Although cranberry is not the only food that contains high levels of fructose, this appears to be the only fruit that has substantial anti-adhesive properties, therefore, proanthocyanidin may be identified as the main component of cranberries which results in its anti-adhesive action. Similar process of bacterial adhesion and aggregation has been observed in the formation of the dental biofilm associated with caries. Also in that setting, cranberry juice was shown to inhibit or reverse the aggregation of oral bacteria by way of an active constituent, highly soluble in water, devoid of proteins, carbohydrates and fatty acids (26), identified as a proanthocyanidin (27).

The double-blind, cross-over study of De Martino et al. (16), similar in design to our study but using the T24 urinary bladder epithelial cells and different *E. coli* strains for the anti-adherence test, showed that different amounts of cranberry extract containing 40 and 120 mg proanthocyanidins significantly and dose-dependently reduced adherence indices by 45% and 62%, respectively, compared to placebo. In that study, any "wash-out" effect was ruled out in that all subjects received the same fluid volumes, and no relevant change of pH was detected. However, it can be noted that the anti-adhesion effect observed in this study with the dose of 36 mg/daily proanthocyanidins is comparable to that obtained with the lower dose used by De Martino et al. (40 mg/daily, with no vitamin C), suggesting that results obtained in our study may be consistently ascribed to the cranberry extract. Another study evaluated urinary antibacterial

adhesion activity *in vitro* after the consumption of 108 and 36 mg of cranberry using the human T24 epithelial cell-line. That study also showed a significant dose-dependent decrease of bacterial adherence in eight healthy female volunteers, in a double-blind, placebo-controlled, crossover study (28).

In our study we did not measure proanthocyanidins in the urine samples, because of the structural complexity of these molecules and the absence of commercial standards. Although the kinetics of proanthocyanidins in humans is not well known, data in healthy volunteers indicate that the administration of 2.0 g of proanthocyanidins-rich grape seed extract determines effective serum concentrations of 10.6 ± 2.5 nmol/l procyanidin B-1, two hours after intake (29). Also experimental data indicate that these compounds are absorbed from the intestinal tract, with 19% of the dose excreted unchanged in the urine after the oral delivery of ^{14}C-labeled grape proanthocyanidins to rats (30).

In conclusion, our findings are consistent with previous observations showing that the intake of cranberry extract in adequate amounts helps to protect the epithelial cells of the bladder from adhesion of uropathogenic *E. coli* strains. A contribution to the clarification of the anti-adherence mechanisms of cranberries can support the concept of a role of cranberry extract in the prevention of urinary tract infections in women by way of removing this virulence factor. The clinical relevance of this dietary supplement is to be assessed in appropriate clinical trials.

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