

Natural Antioxidants and Vitamins Supplementation Shelters Adolescents from Upper Respiratory Tract Infection

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Abstract: *Context:* Several decades of dietary research recommended the consumption of antioxidants and vitamins rich foods as a protective tool against a broad portfolio of diseases

Aims: This study aims to test if oral supplementation of natural antioxidants and vitamins before the winter season, may reduce the occurrence of upper respiratory tract infection (URTI) in adolescents.

Settings and Design: Natural antioxidants and vitamins supplements were given to 90 adolescents (45 males, and 45 females) from an orphanage against a placebo during three months in a double-blind fashion.

Methods and Material: Saliva was collected before and after supplementation. The antioxidant activity of saliva was determined *in vitro* using electrolysis as a free radical generating system. Additionally, total antioxidant activity, glutathione and ascorbic acid levels in the saliva were evaluated before and after supplementation. The URTI frequency was recorded throughout the winter season (3 months).

Statistical Analysis: All values were expressed as means \pm SEM. Significance of the results was assessed using Student's t-test and Fisher's test

Results: Data indicated that only five individuals from the group that received antioxidants and vitamins supplements manifested URTI while 14 adolescents from the non-supplemented group showed symptoms of URTI. Biochemical analysis revealed that the saliva in provenance from the supplemented group exhibited a higher capacity to scavenge free radicals compared to its capacity before supplementation. This supplementation also increased the total antioxidant activity and the levels of both ascorbic acid and glutathione in the saliva.

Conclusions: We concluded that oral intake of antioxidants and vitamins protects against URTI through increased antioxidant activity.

Keywords: Antioxidants, vitamins, saliva, adolescents, upper respiratory tract infection (URTI).

INTRODUCTION

Alternative and complementary medicine is gaining popularity view its focus on natural extracts for the treatment of various diseases. In this regard, numerous epidemiological studies correlated the consumption of adequate amounts of fruits and vegetables with reduced risk of cancer, protection against cardiac events (related to atherosclerosis), improvement of the immune system in low-grade inflammatory diseases, and stabilization of neurological conditions in elderly [1-4]. While studies attributed the beneficial effect of fruits and vegetables consumption to their enrichment in natural antioxidants, others provided doubtful information to this effect [5]. Nonetheless, antioxidants (AOs) are generated by the body (internal anti-

oxidants), but their full activation requires the presence of external cofactors such as trace elements (Zn, Se...). Therefore, vitamins supplement would be beneficial for optimal activity of AOs. In this regards, the consumption of fruits is of essence view their enrichment with vitamins (E, C and A) and other micro-nutrients such as polyphenols [6, 7].

Empirically, a reduction in AOs level was observed in patients with asthma, and those suffering from chronic obstructive pulmonary disease or lymphoma [8]. It has also been reported that premature infants exhibit lower AO levels and therefore are more vulnerable to damage caused by free radicals such as retinopathy, pulmonary diseases, ...etc. [9]. Together, these studies suggest that abnormal levels of antioxidants could be an indicator of several diseases, particularly those related to respiratory dysfunction. To date, the development of AO therapy yielded two significant opinions: the first favouring a preventive

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treatment in mega doses of AO and vitamins, and the second revealed more lenient towards a curative vitamins/AO therapy [10].

In the twentieth century, the quality of food/cuisines has drastically changed, leading to imbalances in the supply of natural materials, particularly AOs and vitamins. Thus, pharmaceutical progresses mainly focused on generating synthesized AO compounds (among other vitamins) while less attention was given to natural antioxidants, particularly in Lebanon. This pilot study aims to investigate the profile of a natural vitamins/AO complex in the protection against upper respiratory tract infection (URTI) in adolescents. We found that the supplementation of natural vitamins/AO complex to a group of adolescents before the beginning of the winter season significantly reduced the development of URTI and promoted the capacity of their saliva to scavenge free radicals and to produce higher levels of AO molecules.

SUBJECTS AND METHODS

Subjects

We selected 100 adolescents (50 boys and 50 girls) aged between 12 and 17 years, living in two buildings within the same orphanage, with no history of

chronic diseases or other abnormalities. All participants were subjected to the same diet and environmental factors. They were randomly divided into two groups of 50 each: one group (G1: 25 boys and 25 girls) received from the beginning of October, and over three months, food supplementation containing natural extracts purchased from Pileje Laboratories, Nice France (Table 1) including antioxidants (AO) and vitamins (1 capsule every two days). Simultaneously, the second group (G2: 25 boys and 25 girls) received the same capsule containing a placebo.

Monitoring of each teenager was conducted under the supervision of the orphanage physician and two assistants, for three months, to ensure a successful implementation of the protocol. Records of the participant's diet and the occurrence of URTI, or other infectious diseases, during winter were maintained. Both weight and height of the candidates were collected every month, and a health assessment was performed to monitor oral or digestive infections. Two saliva samples were collected for each participant; the first collection was made before the oral supplementation of vitamins /AO capsule and the second one at the end of the experiment (after three months). These samples were frozen (-80 °C) until the day of the dosage assay for the level of antioxidants in

Table 1: Nutritional Analysis of the Natural Supplement

| Average values per capsule | | NRV* |
|----------------------------|------------|------|
| Vitamin A (Beta carotene) | 500 µg ER | 62% |
| Vitamin E | 12 mg α-ET | 100% |
| Vitamin C | 80mg | 100% |
| Thiamine (B1) | 1,1 mg | 100% |
| Riboflavin (B2) | 1,4 mg | 100% |
| Niacin (B3) | 16 mg | 100% |
| Vitamin B6 | 1,4 mg | 100% |
| Folic Acid (folates) | 200 µg | 100% |
| Vitamin B12 | 2,5 µg | 100% |
| Zinc | 10 mg | 100% |
| Selenium | 55 µg | 100% |
| Porphyria HSP® | 10 mg | |
| Grapes Polyphenols | 1,8 mg | |
| Lycopene | 2 mg | |

*NRV: Nutrient Reference Values.

Bulking agent: maize starch, vitamins (natural beta-carotene, E, B1, B2, niacin, B6, folates, B12, C), vegetarian capsule, minerals (zinc citrate, yeast cultivated in a selenium-rich environment), tomato lycopene, anti-caking agents: silicon dioxide and magnesium stearate, Porphyral HSP®* (seaweed extract: Porphyra umbilicalis, carrier: maltodextrins), grape marc and grape seed extracts *Vitis vinifera*.

the saliva. Participants that did not adhere with the plan of supplement intake were excluded from the study (5 subjects treated and five placebos).

The Institutional Review Board and Ethics Committee of the Lebanese University, approved the study protocol and the director of the orphanage signed an informed consent form on their behalf, since the participants were underage.

Diagnosis of the Upper Respiratory Tract Infection

The diagnosis of URTI was monitored on a daily check-up by the physician except (All weekdays except for Sunday), and the information was recorded in the health diary by an assistant. The illness symptoms taken into consideration were: sneezing, cough, runny nose, blocked nose, sore throat. One or more symptoms on at least two consecutive days were defined as an episode of illness.

Collection and Preparation of Saliva

Saliva samples were collected in the morning before taking the first capsule and three months later (24 hours after the last capsule). Participants were first asked to rinse their mouth using distilled water and to sit comfortably for 5 min before spitting unstimulated saliva into a plastic tube, five times per min for 5 min. Samples were centrifuged at 4,000g for 10min at 4°C, and the supernatant was collected and stored in small aliquots at - 80 °C.

Electrolysis as Free Radicals Generating System

To measure the amount of free radicals scavenged by the saliva, a physiological solution consisting of: NaCl (137 mM), KCl (2.7 mM), MgCl₂·6H₂O (1 mM), CaCl₂·2H₂O (1.5 mM), NaH₂PO₄·2H₂O (0.4 mM), and NaHCO₃ (12 mM) was used combined to electrolysis. The electrolysis unit is made up of a stimulator adjusted to 10 mA by a sensitive multimeter, wires that connect it with two platinum electrodes which are introduced into a bowl containing 20 ml of the physiological solution, and a magnetic stirrer for rapid mixing and homogenization of the medium [11]. During electrolysis, 1 ml was taken every min, from the physiological solution, and was added to 2 ml of N, N - diethyl-P-phenylenediamine (DPD) (25 mg/ml) in a specific tube that was vortexed. Similarly, the electrolysis of the physiological solution was performed in the absence (control) or presence of 1 ml of saliva samples. The DPD solution develops a pink colour proportional to the free radicals generated that was

measured using spectrophotometry set at an optical density of 514 nm.

Assessment of Glutathione Peroxidase Activity

Glutathione peroxidase (GSHPx) activity was assessed by the use of a commercially available kit from Randox Laboratory Ltd., Ardmore, the UK following the manufacturer's protocol. Briefly, GSHPx activity is evaluated based on the following principle: GSHPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to NADP⁺. The absorbance of the reaction was measured at 340 nm using a spectrophotometer.

Determination of Ascorbic Acid Levels

The concentration of ascorbic acid in each saliva sample was estimated by the ferrozine method. This is a ferrous chromogenic method that depends on the reduction of ferric to ferrous ions by ascorbate, which is simultaneously converted to dehydroascorbate. The ferrous ions formed are subsequently reacted with ferrozine [(3- c2 pyridyl) – 5, 6 – bis (4 – phenol sulphonic acid) 1, 2, 4 triazine]. The chromogenic complex absorbs maximally at 550 nm (Spectrophotometry). Freshly prepared ascorbic acid standards were used to calibrate the assay [11].

Determination of Total Antioxidant Activity (TAA)

The total antioxidant activity of saliva was estimated by the ABTS assay (Randox Lab) that involves the interaction of ferrylmyoglobin radical produced by the activation of metmyoglobin with phenothiazine. ABTS [2,2'-azinobis-(3-ethylbenzo-thialozine-6-sulphonic acid)] produces the ABTS radical cation. This blue chromogene exhibits characteristic absorption at 660 nm (Spectrophotometry). In the presence of antioxidants, the amounts of ABTS radical cation produced are decreased. Thus the absorption is inhibited proportionally to the total antioxidant capacity. The assay was standardized using the vitamin E analogue Trolox [11, 12].

Statistical Analysis

All values were expressed as means ± SEM. Significance of the results was assessed using Student's t-test for unpaired data and set at p<0.05.

Fisher's test was also applied to compare the occurrence of URTI in the treated and untreated groups.

RESULTS

Vitamins/AO Supplement Reduced the Occurrence Rate of URTI in Adolescents

All individuals that completed their participation in this study were medically examined for the occurrence of URTI. Interestingly, a medical examination revealed that only five adolescents (two boys and three girls) had caught URTI in the group supplemented with vitamins/AO (45 individuals), while 14 adolescents in the non-supplemented group who received placebo (45 individuals) were suffering from severe URTI (8 boys and six girls). Fisher's exact test was performed and showed a significant difference between the two above-mentioned groups ($p = 0.0092$). However, there was no significant difference in body weight or other symptoms between these two groups. The data presented in Table 2 summarizes our finding with respect to the URTI results.

Table 2: Upper Respiratory Tract Infection (URTI) Symptoms

| | Placebo | Vitamins/AO | p |
|--------------------------|---------|-------------|------|
| Total URTI symptoms | 14 | 5* | 0.03 |
| Individual URTI symptoms | | | |
| Sneezing | 8 | 3 | 0.19 |
| Cough | 7 | 1* | 0.05 |
| Runny nose | 5 | 2 | 0.43 |
| Blocked nose | 7 | 1* | 0.05 |
| Sore throat | 4 | 1 | 0.36 |

Evaluation of the Antioxidant Levels in Saliva from Vitamins/AO Consumers

We first evaluated the capacity of saliva to scavenge free radicals using electrolysis and the DPD colorimetric assay as detailed in the materials and methods section. Figure 1 shows, in Panel A, the absorbance (at 514 nm) of three samples (A, B, and C) recorded every one minute up to 5 min. Tracing B shows that the saliva submitted to electrolysis before taking the supplement has less ability to scavenge free radicals than the saliva of the same individuals, but after three months of vitamins/AO supplementation (A); the different readings starting of $t=2\text{min}$ are significantly

lower in tracing A compared to B. tracing C represent samples from a physiological solution confirming the generation of free radicals by electrolysis. In contrast, Panel A shows no significant difference in both tracing A and B representing the after and before placebo treatment, respectively. Our results confirm that saliva from adolescents that were subjected to vitamins/AO supplements was able to scavenge free radicals more than the saliva from the same subjects before vitamins/AO consumption (Figure 1).

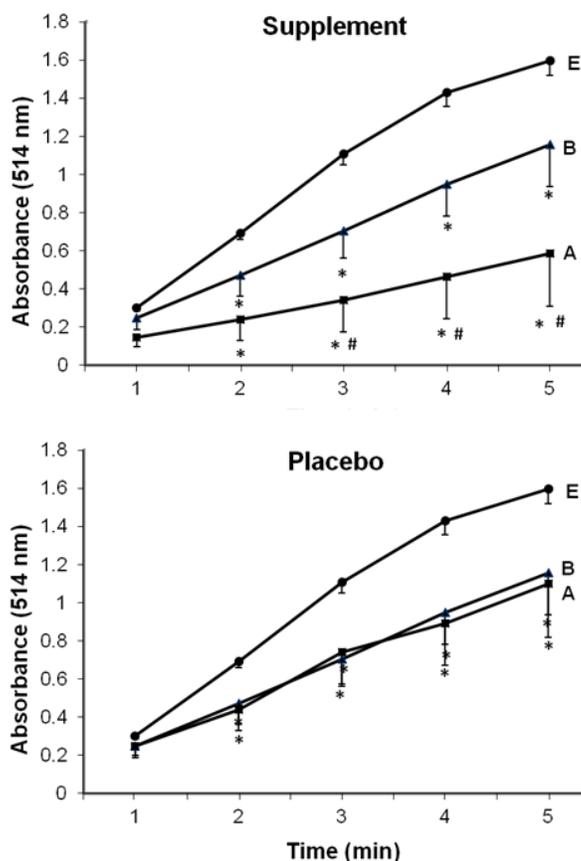


Figure 1: Upper panel: Electrolysis of physiological solution alone (E), or saliva from the group G1 before (B) and after Vitamins/AO supplement (A). Lower panel: Electrolysis of physiological solution alone (E), or saliva from the group G2 before (B) and after placebo supplement (A). * $p < 0.05$ versus E and # $p < 0.05$ versus B using Students T-test, $n=45$.

Glutathione and Ascorbic Acid Levels are Higher in the Saliva of Individuals Subjected to Vitamins/AO Supplement

Both glutathione and ascorbate (ascorbic acid) possess a high capacity to scavenge free radicals. Since our data showed that the saliva from individuals that took the vitamins/AO supplement was able to scavenge free radicals generated by electrolysis, we opted to verify the levels of both glutathione and

ascorbate in the saliva from these individuals. Figure 2 shows that vitamins/AO supplement increased the levels of both glutathione (Panel A) and ascorbate (Panel B) in saliva from individuals that underwent the oral supplement, while that of placebo treatment remained unchanged. This data indicates a higher scavenging capacity of free radicals by the saliva after the consumption of vitamins/AO supplement.

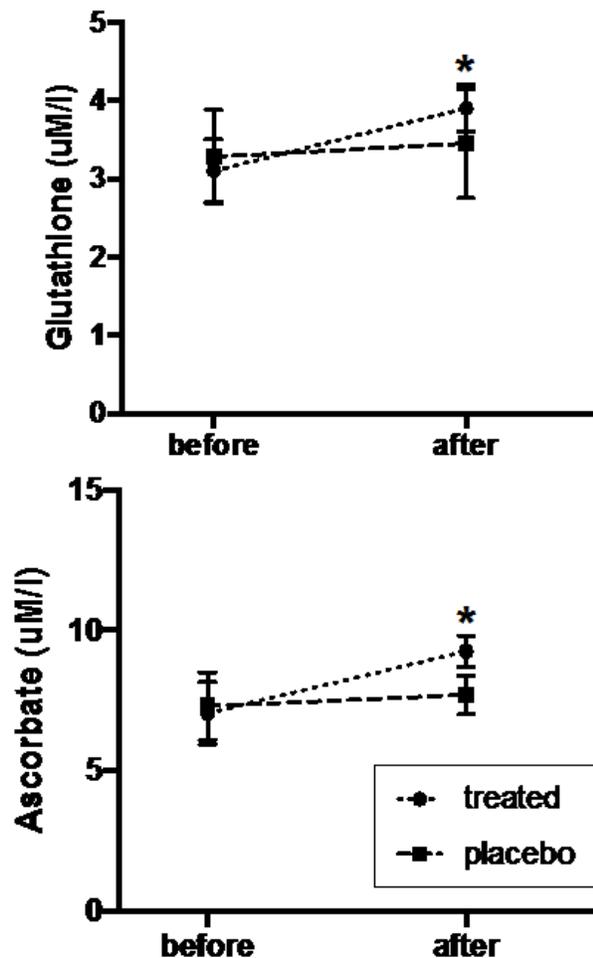


Figure 2: Upper panel: Glutathione levels in saliva of group G1 and G2 before or after three months of supplementation with Vitamins/AO or placebo. Lower panel B: Ascorbate levels in saliva from group G1 and G2 before or after three months of supplementation with vitamins/AO or placebo. * indicates $p < 0.05$ using Student's T-test, $n = 45$.

Vitamins/AO Supplement Increased TAA in the Saliva

Our data showed that the saliva from individuals that were subjected to vitamins/AO supplement exhibited higher free radical scavenging capacity than the one from the placebo group. We also showed that the two significant antioxidants, glutathione and ascorbate, were higher in the saliva of these subjects. However, since the scavenging capacity of the saliva

can be a function of other molecules, we opted to evaluate the total antioxidant activity (TAA) of the saliva in these candidates. Figure 3, reveals that individuals who underwent the vitamins/AO treatment showed higher TAA in their saliva compared to the placebo group. This is indicative that the vitamins/AO supplementation generated a powerful scavenging ability of antioxidants in the saliva.

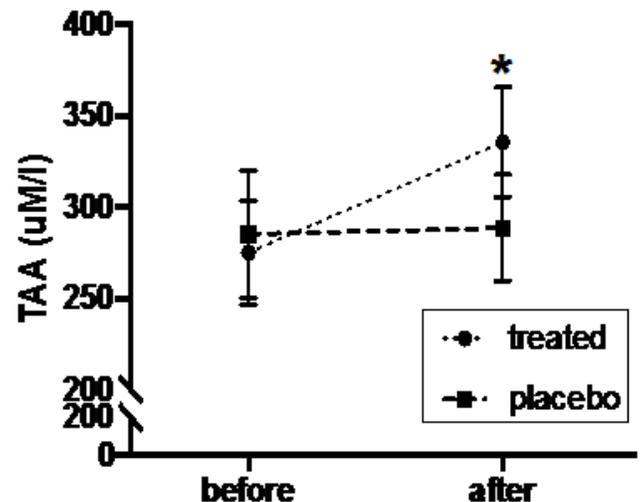


Figure 3: Total antioxidant activity (TAA) levels in saliva from group G1 and G2 before or after three months of supplementation with Vitamins/AO or placebo. * indicates $p < 0.005$ using Student's T-test, $n = 45$.

DISCUSSION

Our study provides new insights on the role of natural extracts supplement as a preventive treatment against influenza. We found that supplementation of natural extracts (vitamins/AO) before the beginning of the winter season protects against URTI symptoms. This was associated with increased levels of salivary antioxidants (Glutathione and ascorbate) as well as the total antioxidant activity (TAA) of the saliva in provenance from candidates subjected to vitamins/AO supplement.

Infections of the throat (larynx), the main airway (trachea), or the airways going into the lungs (bronchi) are common. These infections are usually called laryngitis, tracheitis, or bronchitis. We used the term URTI to include any, or all, of these infections. Most URTIs are due to a viral infection. Cough is usually the main symptom. Other symptoms include fever, headache, aches and pains. Cold symptoms may occur if the infection hits the nose. Symptoms typically peak after 2-3 days, and then gradually disappear. The cough may persist after the infection has gone; this is because inflammation of the airways may take a while

to settle. It may take up to 2-3 weeks after other symptoms have gone, for a cough to clear completely.

Vitamin supplements have been adopted as a preventive strategy to fight against the development of flu. While the immune system is programmed to defend our body against external aggressions, the effectiveness of this system is dependent on the nutritional status and other factors that interfere with its self-functioning: stress, negative attitude, depression, pollution, tobacco... [13-15]. In this context, free radicals play a significant role in the pathogenesis of various disorders, including inflammation, rheumatoid arthritis, asthma ... etc, leading to oxidative stress. Oxidative stress may be defined as an imbalance between the production of free radicals and the antioxidant defence mechanisms, in the cell. Moreover, free radicals (e.g., superoxide radical, peroxyxynitryl, hydroxyl radical and hydrogen peroxide) are constantly produced as a result of metabolic reactions in living systems [16].

Based on the results we obtained in our experimental conditions, it is highly suggestive that a non-exaggerated supplementation (one capsule every two days) of natural extracts (including multivitamins and antioxidants), helps protect against free radicals attack, particularly at the salivary level. In fact, the saliva, long known for its protective effect and its lubricating properties, is now being increasingly used in parallel with blood tests for the diagnosis of many oral diseases particularly those related to smoking and/or alcohol consumption. This promoted the development of saliva test kits that offer the same sensitivity as that of blood test kits in a non-invasive manner.

The advantages of evaluating saliva seem to be undeniable for several reasons. In fact, saliva can be collected noninvasively without violating the privacy of candidates; this is done under the visual control of medical personnel and investigators. In addition, working with saliva provides a unique tool to perform repetitive readings over time for kinetic studies as well as systematic evaluations in a population setting through epidemiological screening. This allows for the real-time identification of compounds, and the correlation between both the salivary and blood concentrations of these compounds [17]. It is true that salivary assays are not conventionally used to evaluate metabolic changes; however, saliva is a diluted plasma and substances from the blood can easily end up in saliva and faithfully reflect their plasma concentration.

Furthermore, plasma hormones and some elements are found in either bound or unbound state in the blood; however, in saliva, only their unbound form is found. Therefore, it would be more accurate to assay these substances in saliva. In addition, scientific studies have shown a correlation between plasma and saliva for most hormones and enzymes [18-20]. Thus, the increased capacity of saliva to scavenge free radicals *in vitro* reflects the antioxidant capacity of the plasma in these candidates. In addition to increased levels of glutathione and ascorbate in the saliva of treated individuals, we assessed the total antioxidant activity to confirm that the saliva's capacity to scavenge free radicals is spread beyond the effect of glutathione and ascorbate.

Over the years, vaccination has proven insufficient to prevent infection, particularly the one against influenza entirely. In fact, only one out of four (1/4) vaccinated people develop protection against influenza [21]. From the results obtained, it can be concluded that supplementation with natural vitamins/AO improved the antioxidant capacity in teenagers. The role of vitamins A, C and E is well known in scavenging free radicals [22-24] however, types B of vitamins, that are present in the multivitamin capsule, indirectly promote the antioxidant capacity through the metabolism of GSH [25-27]. The clinical profile of the study is as important as the biochemical profile. Thus, the significant reduction of flu symptoms among the supplemented group is a tangible evidence of the efficiency of antioxidants supplements in enhancing the immune capacity against a widespread viral infection like influenza.

CONCLUSION

In conclusion, natural vitamins/AO supplementation to adolescents prevents the development of URTI during the winter season. The total antioxidant capacity of the organism, as measured in saliva, markedly increases after the supplementation is associated with reduced URTI. Further work is warranted to adopt our proposed multivitamin supplementation as a preventive therapy for influenza.

KEY MESSAGE

Antioxidant therapy has been proposed against body infections, but the efficiency depends on the dose acting on redox state. In our experimental conditions, a light supplementation with multivitamins and natural antioxidants before winter season appears to protect

against flu symptoms in adolescents. Antioxidant biomarkers in saliva have been measured in support.

RESEARCH FUNDS

Doctoral School, Lebanese University.

CONFLICT OF INTEREST

None.

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