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Effects of ascorbic acid on anxiety state and affect in a non-clinical sample

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Objective Given that the literature data indicates that ascorbic acid may have an anxiolytic effect, we hypothesized that a single oral administration of ascorbic acid could acutely affect emotional states. Methods The effects of acid ascorbic supplementation on anxiety and other emotional states were evaluated by the State-Trait Anxiety Inventory (STAI), and Visual Analogue Mood Scale (VAMS). Immediately before, and 2 hours after receiving a single ascorbic acid dose (1000 mg) or placebo, 142 graduate students were evaluated by the STAI and VAMS in a randomized, double-blind, placebo-controlled trial. Results No changes from basal levels were observed in the STAI state-anxiety or VAMS scores. However, the ingestion of ascorbic acid by the 25% more anxious healthy subjects (women; 14 control and 23 ascorbic acid), as defined by the STAI trait-anxiety scale, produced a significant reduction from baseline anxiety scores in the STAI state-anxiety scale and VAMS anxiety subscale. The study evaluated a small sample with narrow sociodemographic characteristics, composed mainly of healthy young females (> 94%) enrolled in post-graduation courses, without controlling diet, physical activity, and formal psychiatric diagnosis. Conclusions: Despite the sample size limitation, this study provides the first evidence of an acute anxiolytic effect of ascorbic acid. Broader population studies are required to evaluate the clinical relevance of presented data.

Key words: ascorbic acid, anxiety, trait anxiety, State-trait Anxiety Inventory (STAI), Visual Analog Mood Scale (VAMS)

INTRODUCTION

Anxiety is characterized by apprehension, worry and tension that may be understood as a physiological, adaptive response to potential threats (Perusini and Fanselow 2015). However, persistent or exaggerated anxiety is pathological and constitutes the hallmark of anxiety disorders, which are the most common psychiatric disorders, with a lifetime prevalence of almost 30% (Greenberg et al. 1999, Kessler et al. 2005, Viana and Andrade 2012). Anxiety disorders are classified by the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-V) in separation anxiety disorder, selective mutism, specific phobia, social phobia, panic disorder, agoraphobia, and generalized anxiety disorder (American Psychiatric Association 2013). These disorders are more frequent in women than in men (Asher et al. 2017, Li and Graham 2017), and are well known to impair quality of life and performance at work, being highly costly to society (Baxter et al. 2014). Clinically relevant anxiety is also present in a myriad of mental illnesses, particularly mood disorders. For example, comorbidity between major depression and anxiety disorders occur in more than 50% of patients (Kessler et al. 2003). Of note, anxiety disorders are also often associated with hyperthyroidism and inflammatory diseases such as rheumatoid arthritis, irritable bowel syndrome, diabetes, and asthma (Culpepper 2009, Hajebrahimi et al. 2016). Anxiety can be studied within a state-trait paradigm, in which trait anxiety represents a generalized, enduring individual predisposition to experience anxiety, while state anxiety consists of acute, transitory emotion (Endler and Kocovski 2001).

Antidepressants (especially selective serotonin and norepinephrine reuptake inhibitors) are the first line treatment for several anxiety disorders (Baldwin et al. 2014, Bandelow et al. 2015, Katzman et al. 2014). Although effective in many patients, these drugs are not free of side effects, which include mainly metabolic, sexual, and cognitive dysfunctions (Masand and Gupta 2002, Uzun et al. 2010). Antidepressants typically

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take several weeks to produce a therapeutic response and less than two thirds of patients show significant improvement in symptoms (Bystritsky 2006). The scientific and pharmaceutical community (Farach et al. 2012) has avidly sought alternative drug regimen and novel drugs, able to produce a faster therapeutic response without serious side effects in larger proportions of patients.

Along the years, several drugs were incorporated in the repertoire for anxiety disorders therapies. The use of benzodiazepines is growing in the anxiety therapy, however, tolerance and dependence frequently develop (Gravielle 2016), and their long-term prescription is controversial and known to be problematic in specific populations, such as the elderly and individuals with a history of substance abuse (Cloos and Ferreira 2009, Dell'osso and Lader 2013, Starcevic 2014). Recently, the use of pregabalin was included in the guidelines for the treatment of generalized anxiety disorder as the first line treatment (Katzman et al. 2014), or an alternative first line treatment (Baldwin et al. 2014). Pregabalin is an anticonvulsant drug that binds to the $\alpha_2\delta$ subunit of the voltage-dependent calcium channel in central nervous system, decreasing the release of neurotransmitters, which includes glutamate (Martinotti et al. 2013). Pregabalin is also able to increase the activity of glutamate transporter type 3, thus clearing glutamate from the synaptic cleft (Ryu et al. 2012).

Ascorbic acid is known as vitamin C, a water-soluble antioxidant vitamin, essential to human beings and to several other mammals. The anion form (ascorbate) is the active form of vitamin C, known to have free radical scavenging activity, thus reacting with free radicals, such as protein thiyl radical, protecting biomolecules from oxidative damage (Nauser et al. 2015). Vitamin C also participates indirectly as an antioxidant by regenerating vitamin E, contributing to antioxidant protection against lipid peroxidation and in the elimination of free radicals (Jiang 2014, Traber 2007). Nevertheless, in presence of transition metals (Schoenfeld et al. 2017) or under decreased levels of glutathione (Nauser et al. 2015), ascorbic acid can behave as an oxidative molecule, increasing the production of oxygen free radicals. This property has been recently explored to kill cancer cells (Schoenfeld et al. 2017).

Ascorbic acts as a cofactor for several enzymes (Lane and Richardson 2014). Scurvy has been associated to psychiatric symptoms (Carr and Vissers 2012, DeSantis 1993, Kinsman and Hood 1971), and several preclinical and clinical studies indicate ascorbic acid has effects on cognition and emotion, which seem to be unrelated to its classical role as a vitamin. Of note, ascorbic acid modulates glutamatergic function (Majewska et al. 1990, Moretti, et al. 2012b), and exhibits

antidepressant activity in different rodent models of depression (Moretti, et al. 2012a, 2012b, 2015) through a mechanism dependent on mTOR activation, similar to ketamine (Moretti et al. 2014). There is scarce information in the literature regarding a potential anxiolytic effect of ascorbic acid. A few studies on non-clinical samples have shown that ascorbic acid can produce improvement in mood and relieve on depressive and anxiety symptoms (Brody 2002, Khajehnasiri et al. 2013, de Oliveira et al. 2015). Ascorbic acid can be effective in patients with major depression, generalized anxiety disorder and general medical conditions (Amr et al. 2013, Gautam et al. 2012, Mazloom et al. 2013, Zhang et al. 2011). These studies have focused on the effects of ascorbic acid administered over a period of weeks to months while the short-term effects of a single dose of ascorbic acid on emotional states are unknown.

The exact mechanisms associated with the putative anxiolytic effects of ascorbic acid are unknown. Preclinical studies include a series of possible targets, such as antioxidant and neuroprotective action (Moretti et al. 2012a, 2012b), and mechanisms related to the antidepressant action of ascorbic acid (Binfaré et al. 2009, Lopresti 2015, Majewska et al. 1990, Moretti et al. 2012a, 2012b, 2014, 2015, Yusa 2001), which can potentially be explored to treat anxiety. The signaling pathways that could be implicated in the antidepressant effect of ascorbic acid effects include activation of phosphoinositide 3-kinase/mammalian target of rapamycin pathway (Moretti et al. 2014). These modulatory pathways include potassium channels (Moretti et al. 2012a), NMDA receptors, activation of monoaminergic systems (Binfaré et al. 2009), as well as pathways involving nitric oxide (Majewska et al. 1990, Yusa 2001, Zomkowski et al. 2012). Interestingly, NMDA administration to mice completely reversed the antidepressant-like effect of ascorbic acid, while the combined administration of ascorbic acid and a sub-effective dose of MK-801, an NMDA receptor antagonist, presented synergistic antidepressant-like effect in mice (Moretti et al. 2012b).

In this context, the present study aimed at investigating the effects of ascorbic acid on anxiety state and mood in a sample of graduate students through a double-blind, randomized, placebo-controlled design. Self-report measures of anxiety and affect were applied before and after the oral administration of a single dose of ascorbic acid or placebo. In addition, taking into account the possibility that ascorbic acid may elicit an anxiolytic effect particularly in anxious subjects; a secondary analysis was performed subdividing the sample into two groups according to the anxiety background, as evaluated by State-Trait Anxiety Inventory (STAI) Trait score.

METHODS

Participants

The Human Research Ethics Committee of the Federal University of Santa Catarina approved the protocol (number 1147315), and the investigation was carried out in accordance with the latest version of the Declaration of Helsinki. Informed consent of participants was obtained after procedures had been fully explained A sociodemographic questionnaire, including questions about the current use of psychotropic medications was also presented to participants. Participants were graduate students of both genders but mostly constituted of women (>95%), recruited in *lato sensu* post-graduation courses in the cities of Florianópolis and São Paulo, Brazil. Subjects who reported allergy related to the use of ascorbic acid preparations, pregnancy, history of acute of chronic renal failure, or regular supplementation with ascorbic acid were not included in the study. Regular diet and physical activity, and psychiatric diagnosis, were not controlled. Some individuals (5–8%) did not fill the post-treatment forms, 8 individuals for the STAI and 10 for the VAMS, thus were excluded from the analysis. Missing values are detailed in the legend to Table I. From 144, six individuals in the control and two in the ascorbic acid group reported psychotropic use, and 27% in each group reported chronic diseases.

Psychological measures

Anxiety and affect were assessed by the self-report measures STAI (Spielberger et al. 1970) and VAMS (Norris 1971). Both questionnaires were adapted and validated in Brazil (Biaggio et al. 1977, Gorenstein and Andrade 1996, Zuardi et al. 1993, Zuardi and Karniol 1981). The STAI is comprised by the anxiety state (S-anxiety) and the anxiety trait (T-anxiety) scales, each one including 20 items with 4 Likert responses; score ranges from 20 to 80; the higher the scores the higher the anxiety trait or state. The VAMS is an analog scale comprised by 16 pairs of adjectives of opposite feelings separated by a 100 mm line; the respondent is instructed do mark the line according to how he or she feels at the time of assessment. The adjectives represent extreme feelings and can be generally regarded as "positive" or "negative" (e. g. strong-feeble, happy-sad, contented-discontented, interested-bored). A mean overall score can be calculated – the higher the measure the higher the "negative" feelings – as well as dimensions of anxiety, physical sedation, mental sedation, and other feelings.

Depressive and anxious symptoms were evaluated using the Brazilian version of the Hospital Anxiety and Depression Scales (HADS) (Botega et al. 1998, Zigmond and Snaith 1983). The HADS is comprised by the anxiety subscale and the depression subscale, each one with 14 items with 4 responses; scores in subscales range from 0 to 21.

Procedures

The study has a double-blind, randomized, placebo-controlled design. Participants were informed about the objective of this work and were aware about the existence of ascorbic acid or vehicle in capsules and that researchers did not have access to this information. Participants were asked to avoid drinking tea, coffee or other stimulating substances before testing. As vitamin C has a sour taste, participants were instructed to avoid chewing the capsules, thus remaining blind regarding the experimental group they belong to. Authors are unware if any participant did not respect this instruction, and this is a limitation of our work. The known side effects of ascorbic acid was explained in the consent form. While participants were attending their weekend post-graduation classes, trials were performed in the morning in the student's classroom during a class interval and participants were instructed not to intake any food, drink or dietary supplements during a two-hour period, and not have ingested ascorbic acid for at least six hours before the test. Around 10: 00 a.m., and immediately before the administration of ascorbic acid or placebo, participants answered the VAMS, STAI S-anxiety and T-anxiety scales, and HADS. Thereafter, students in a given row received two gelatin capsules containing 500 mg ascorbic acid each (Hebel Welcome, China), and the students in the next row received two gelatin capsules containing cornstarch. Water in a disposable cup was provided along the capsules. The procedure was repeated for the next two rows, and so, in a way that groups were balanced in size for the two groups, ascorbic acid and placebo. Starting row and starting groups were alternated each other class. After two hours, students answered VAMS and STAI S-anxiety scale. Capsules were identified by a numerical code and researchers who administered them were blind to their content, and groups were only disclosed after compilation and analysis of all data.

Data analyses

Changes from baseline emotional states were defined by differences between the scores in the VAMS and S-anxiety scale. For each of these scales and the factor subscales of VAMS, the score after treatment mi-

Table I. Overall baseline characteristics of the sample, grouped as low-to-moderate trait anxiety or as high trait anxiety, according to State-Trait Anxiety Inventory (STAI) Trait anxiety score. High trait anxiety was defined as the group presenting Trait-STAI scores equal or higher than the 75th percentile of the sample, while the remaining of the sample was included in the low-to-moderate trait anxiety group

Variables[a,b]	All subjects (n=144)					Low-to-moderate trait anxiety subjects (n=107)					High trait anxiety subjects (n=37)				
	Placebo	n	Ascorbic acid	n	p	Placebo	n	Ascorbic acid	n	p	Placebo	n	Ascorbic acid	n	p
Age (years)	27.0 (25.0–30.8)	68	29.0 (26.0–32.5)	73	0.08	27.0 (25.0–30.3)	54	29.0 (26.0–33.0)	50	0.04	28.1±3.7	14	29.0±5.8	23	0.61
Sex															
Female	67 (95.7%)		73 (98.6%)		0.36	53 (94.6%)		50 (98.0%)		0.62	14 (100%)		23 (100%)		-
Male	3 (4.3%)		1 (1.4%)			3 (5.4%)		1 (2.0%)			0 (0.0%)		0 (0.0%)		
Psychotropic use[c]															
No	64 (91.4%)		72 (97.3%)		0.16	53 (94.6%)		50 (98.0%)		0.62	11 (78.6%)		22 (95.7%)		0.14
Yes	6 (8.6%)		2 (2.7%)			3 (5.4%)		1 (2.0%)			3 (21.4%)		1 (4.3%)		
Chronic disease[d]															
No	50 (72.5%)		54 (73.0%)		0.95	41 (74.5%)		37 (72.5%)		0.82	9 (64.3%)		17 (73.9%)		0.53
Yes	19 (27.5%)		20 (27.0%)			14 (25.5%)		14 (27.5%)			5 (35.7%)		6 (26.1%)		
HADS (score)															
Anxiety	6.8±3.8	69	7.6±4.4	72	0.25	5.9±3.2	55	5.5±2.9	50	0.53	10.4±3.8	14	12.3±3.2	22	0.12
Depression	4.0 (2.0–6,0)	70	5.0 (2,3–7,0)	72	0.21	4.0±2.8	56	3.9±2.7	50	0.83	6.0±3.5	14	8.1±3.5	22	0.10
VAMS (mm)															
Score	614.0±224.0	56	648.7±258.3	62	0.44	591.4±209.9	46	545.0±210.6	43	0.30	717.2±268.0	10	883.4±196.8	19	0.07
Anxiety	133.8±69.3	56	143.9±68.8	62	0.43	126.6±64.1	46	115.3±53.9	43	0.37	167.0±85.4	10	208.7±53.8	19	0.12
Physical sedation	260.6±113.9	56	285.8±132.1	62	0.27	248.8±109.6	46	237.1±104.0	43	0.61	315.0±123.5	10	396.1±124.2	19	0.11
Mental sedation	99.6±39.9	56	102.3±45.5	62	0.73	99.8±40.9	46	93.0±41.9	43	0.44	98.6±37.0	10	123.3±47.3	19	0.16
Other feeling	120.0±64.0	56	116.7±71.2	62	0.80	116.2±62.0	46	99.7±63.1	43	0.21	137.1±73.7	10	155.4±75.1	19	0.54
STAI (score)															
Trait anxiety	43.2±9.2	70	44.9±10.8	74	0.31	39.7±6.1	56	39.0±6.0	51	0.57	57.4±4.9	14	58.0±6.7	23	0.74
State anxiety	40.3±8.3	62	41.3±9.8	65	0.54	38.6±7.1	49	37.1±6.6	45	0.30	46.8±9.5	13	50.7±9.3	20	0.26

HADS, Hospital Anxiety and Depression Scale. VAMS, Visual Analogue Mood Scale. Data missing for the following variables: age, 3 missings; chronic disease, 1 missing; HADS depression, 2 missing; VAMS, 26 missings; STAI state anxiety, 17 missings. [b] Continuous variables with a normal distribution, according to the Kolmogorov-Smirnov test, were analyzed using the Student t test, and shown as mean ± SD. Continuous variables with a non-normal distribution were analyzed using the Mann-Whitney U test, and shown as median (interquartile range). Categorical variables were analyzed using the Fisher exact test. [b] Missing data: age 3 subjects; chronic disease 1 subject; HADS anxiety 3 subjects; HADS depression 2 subjects; VAMS 27 subjects; STAI state anxiety 9 subjects. [c] Self-reported use of antidepressants or benzodiazepines (see text for details). [d] Self-report of any chronic non-psychiatric disease (see text for details).

nus the score before treatment was computed. Further analyses were carried comparing placebo and ascorbic acid groups according to a cut-off score of 75% in the STAI T-anxiety scale: individuals scoring in the 75% lower percentile were defined as "low-to-moderate trait anxiety", while individuals presenting the 25% highest scores were denominated as "high trait anxiety" (Table I). "Low-to-moderate trait anxiety" placebo group was composed of 95% females, while the ascorbic acid was 98%. Placebo group presented 5.4% and ascorbic acid 2% of individuals reporting psychotropic use, while chronic diseases rated 25.5% and 27.5%, respectively, among the "low-to-moderate trait anxiety". In the "high trait anxiety" group, 100% were female, three individuals in placebo and one in the ascorbic acid group reported psychotropic use, while chronic diseases amounted to 35.7% in placebo and 26.1% in the ascorbic acid group. No differences were found between placebo and ascorbic acid regarding gender, psychotropic use, and chronic diseases.

By repeating statistical analysis in subgroups (low-to-moderate trait anxiety and high trait anxiety), these additional comparisons using the same sample increases the chances of false positive results. Alike, comparison of subscales in the VAMS questionnaire results in testing a family of closely related hypotheses. These are possible weaknesses of our work, needing to be taken in account when interpreting results.

Continuous variables were compared using the unpaired t-test or Mann-Whitney U test according to the normality of data, as defined by the Kolmogorov-Smirnov test. Categorical variables were analyzed using Fisher exact test. A significance level of p less than 0.05 was set for all statistical tests. Data was analyzed using the SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) and graphics were plotted using GraphPad Prism 6 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

The demographic and baseline characteristics of participants are summarized in Table I, while Table II present average values and standard deviation after ingestion of placebo or ascorbic acid. In addition to the treatment groups, sample was secondarily ranked as "low-to-moderate", and "high trait anxiety", as defined by a STAI T-anxiety scale score higher than or equal to 50 (corresponding to 25% of the sample). Basal levels are presented in Table I, while Table II present values 2 hours after placebo or ascorbic acid ingestion. Placebo and ascorbic acid groups did not differ at baseline in terms of: gender, self-reported use of psychotropic medications (antidepressants and benzodiazepines, other psychotropic medications were not reported), and scores in the HADS anxiety and depression subscales, T-anxiety and S-anxiety scales, and VAMS and its factor subscales. Subjects spontaneously reported no adverse effects attributable to ascorbic acid. Subjects within the low-to-moderate trait anxiety group,

Table II. Visual Analogue Mood Scale (VAMS) and State-Trait Anxiety Inventory (STAI) State anxiety scores of the sample, grouped as low-to-moderate trait anxiety or as high trait anxiety, according to STAI Trait anxiety score after placebo or ascorbic acid ingestion. High trait anxiety was defined as the group presenting Trait-STAI scores equal or higher than the 75th percentile of the sample, while the remaining of the sample was included in the low-to-moderate trait anxiety group

Variables[a, b]	All subjects (n=144)				Low-to-moderate trait anxiety subjects (n=107)				High trait anxiety subjects (n=37)			
	Placebo	n	Ascorbic acid	n	Placebo	n	Ascorbic acid	n	Placebo	n	Ascorbic acid	n
VAMS (mm)												
Score	590.0±223.1	56	602.0±243.9	62	569.9±198.3	46	563.1±240.8	43	682.4±309.7	10	689.9±233.5	19
Anxiety	127.9±60.6	56	123.0±63.1	62	120.2±58.7	46	112.0±58.7	43	163.2±59.6	10	147.7±67.5	19
Physical sedation	254.5±109.9	56	271.0±125.9	62	244.0±98.4	46	250.5±121.4	43	302.7±149.0	10	317.5±126.7	19
Mental sedation	84.6±41.4	56	91.2±48.8	62	84.2±38.3	46	91.7±49.8	43	86.7±56.1	10	90.2±47.6	19
Other feeling	122.9±67.3	56	116.7±62.6	62	121.4±60.9	46	108.9±58.6	43	129.8±95.5	10	134.5±69.2	19
STAI												
State anxiety score	40.3±8.3	62	41.3±9.8	65	38.6±7.1	49	37.1±6.6	45	46.8±9.5	13	50.7±9.3	20

[a] All variables had a normal distribution according to the Kolmogorov-Smirnov test and are shown as mean ± SD. [b] Data missing for the following variables: VAMS, 26; STAI state anxiety, 17.

who received ascorbic acid, were older than those who received placebo, with a mean ± standard deviation of the mean (SD) of 28.6±6.1 and 30.0±6.3 years (p=0.03).

Fig. 1 depicts changes from baseline in the STAI S-anxiety scale: scores taken two hours after treatment minus scores immediately before treatment. There was no change from the baseline value difference (mean±SD, n) between placebo (-0.0±6.3, *62*), and ascorbic acid (-1.7±9.3, *65*), p=0.24 (Fig. 1A). Analysis of subjects with low-to-moderate trait anxiety also revealed no differences between placebo (-0.2±6.2, *49*), and ascorbic acid (0.5±7.5, *45*) groups, p=0.60 (Fig. 1B). However in subjects with high trait anxiety, the scores in the ascorbic acid group was significantly lower (-6.6±11.2, *20*) than placebo group (0.85±7.0, *13*), p=0.04 (Fig. 1C).

Fig. 2 shows the VAMS analyses and its factor subscales: anxiety, physical sedation, mental sedation, and other feelings. When changes from baseline were analyzed, taking into account the scores in millimeters, two hours after treatment minus scores immediately before treatment, (Fig. 2A), no differences were observed between placebo and ascorbic acid group (mean ± SD, *n*). VAMS: placebo 24.0±220.9, *56*, ascorbic acid -46.7±253.7, *62*, p=0.61; and subscales Anxiety: placebo -5.9±58.2, ascorbic acid 21.0±68.5, p=0.20; Physical sedation: placebo -6.1±105.2,

ascorbic acid -14.8±130.3, p=0.69; Mental sedation: placebo -14.9±55.8, ascorbic acid -11.3±56.3, p=0.71; Other feelings: placebo 2.9±52.7; ascorbic acid 0.01±65.8, p=0.79.

Analysis of subjects with low-to-moderate trait anxiety (Fig. 2B) also revealed no change in the baseline VAMS score (mean±SD, *n*) between placebo (-21.5±208.0, *46*), and ascorbic acid (18.4±235.8, *43*) groups p=0.40. The same was observed in the VAMS subscales: Anxiety: placebo -6.4±59.0; ascorbic acid -3.3±60.5, p=0.81; Physical sedation: placebo -4.8±99.3; ascorbic acid 13.4±113.4, p=0.42; Mental sedation: placebo -15.6±56.0, ascorbic acid -1.3±50.1, p=0.21. Other feelings: placebo 5.2±46.1, ascorbic acid 9.2±67.6, p=0.74.

Interestingly, subjects in the ascorbic acid group, in the sub-set sample with high trait anxiety (Fig. 2C), presented significantly lower VAMS score in the anxiety factor subscale (mean±SD, *n*): placebo -3.8±57.6, *10*, ascorbic acid -61.0±70.1, *19*, p=0.04 (Fig. 2C). The VAMS overall score and the scores in other factor subscales did not differ between placebo and ascorbic acid group. VAMS: placebo -35.3±285.9, ascorbic acid -193.5±235.9, p=0.12; Physical sedation: placebo -12.3±135.2, ascorbic acid -78.6±146.0, p=0.24; Mental sedation: placebo -11.9±57.5, ascorbic acid -33.2±64.5, p=0.39; Other feelings: placebo -7.3±79.0, ascorbic acid -20.8±57.8, p=0.60.
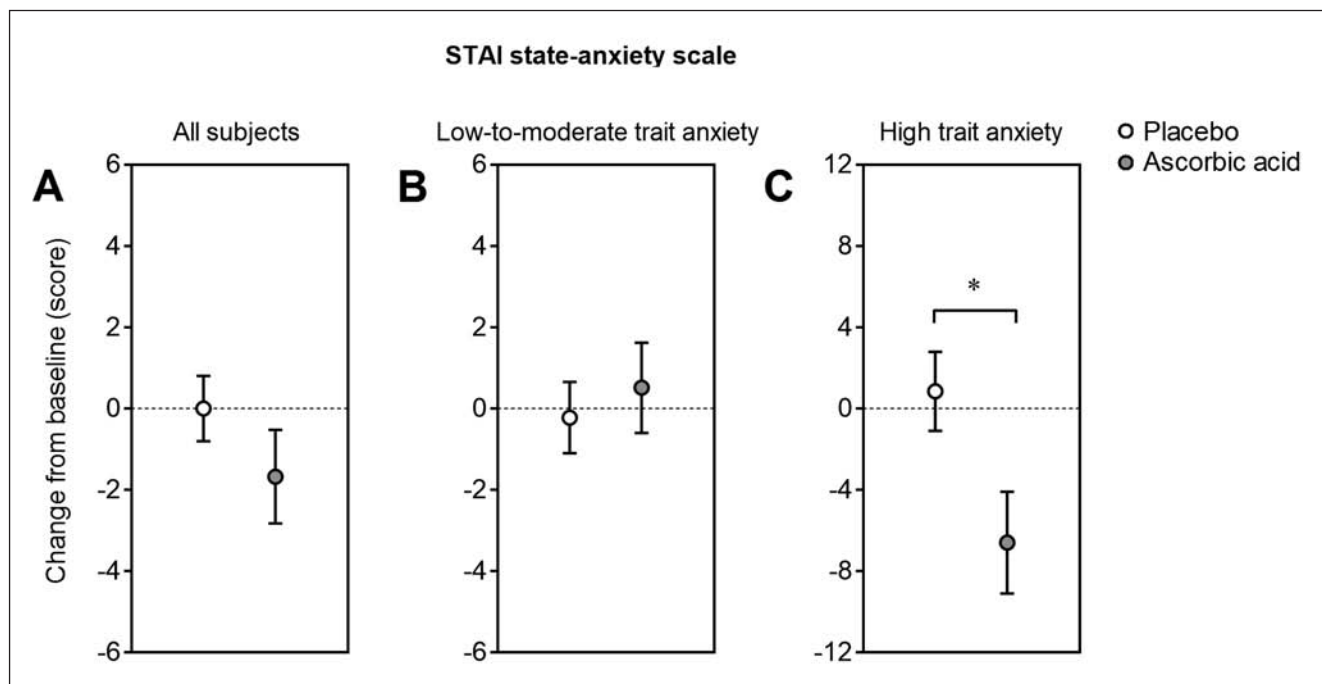

Fig. 1. Effects of ascorbic acid on anxiety state evaluated by the State-Trait Anxiety Inventory (STAI). Immediately before the oral administration of placebo or 1000 mg of ascorbic acid STAI state-anxiety scale was applied, and was reapplied two hours later. Changes from baseline scores are shown as mean and standard error of the mean. Low-to-moderate and high trait anxiety subjects were defined by a score in the STAI trait-anxiety scale applied at baseline lower or higher than or equal to the 75th percentile of the sample. Analyses of all subjects (n=62 and 65 for placebo and ascorbic acid groups, respectively) and subjects with low-to-moderate trait anxiety (n=49 and 45 for placebo and ascorbic acid groups, respectively) revealed no differences between the groups (p > 0.05). Analyses of subjects with high trait anxiety (n=13 and 20 for placebo and ascorbic acid groups, respectively) showed greater reduction of STAI state-anxiety scores in the ascorbic acid group (*p=0.04), as evaluated by unpaired t-test.
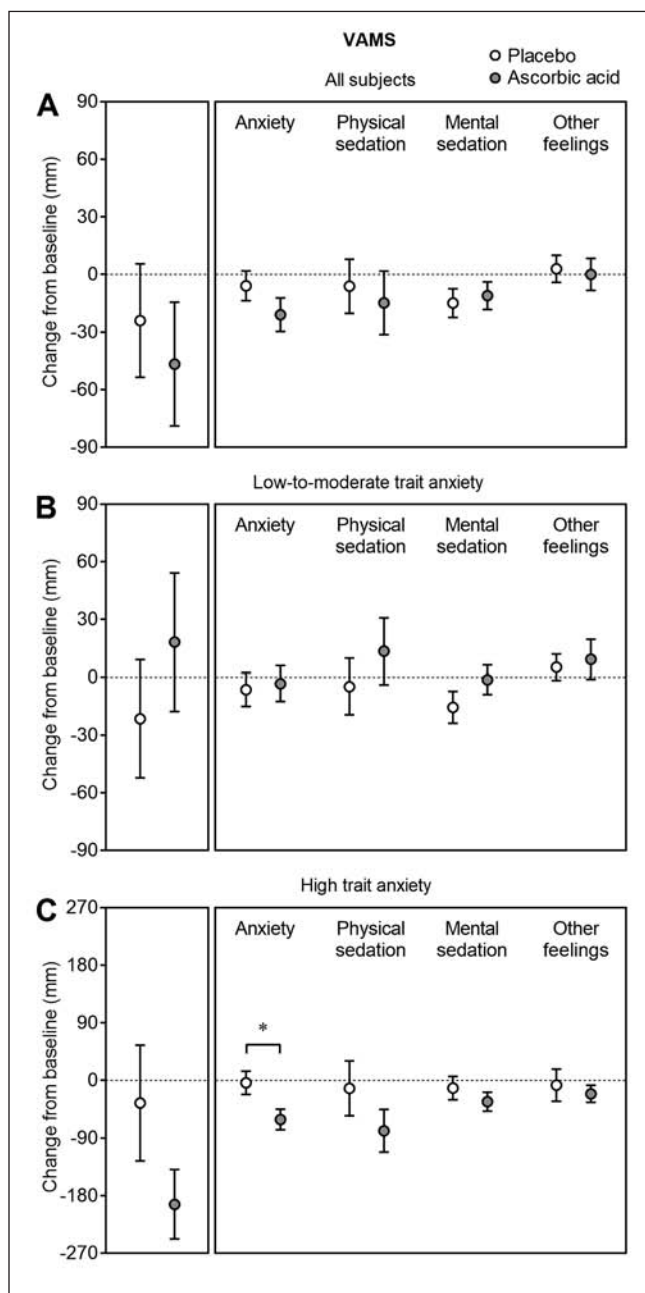
Fig. 2. Effects of ascorbic acid on anxiety state by the Visual Analog Mood Scale (VAMS). Immediately before the oral administration of placebo or 1000 mg of ascorbic acid VAMS was applied, and was reapplied two hours later. Changes from baseline scores are shown as mean and standard error of the mean. Low-to-moderate and high trait anxiety subjects were defined by a score in the STAI trait-anxiety scale applied at baseline lower or higher than or equal to the 75th percentile of the sample. Analysis were performed using the overall score of the VAMS and scores in the factor subscales anxiety, physical sedation, mental sedation and other feelings. Analyses of all subjects (n=56 and 62 for placebo and ascorbic acid groups, respectively) and subjects with low-to-moderate trait anxiety (n=46 and 43 for placebo and ascorbic acid groups, respectively) revealed no differences between groups (p > 0.05). Analyses of subjects with high trait anxiety (n=10 and 19 for placebo and ascorbic acid groups, respectively) showed greater reduction in the anxiety factor scores in the ascorbic acid group (*p=0.04), as evaluated by unpaired t-test.

DISCUSSION

The present study employed a double-blind, randomized, placebo-controlled design to evaluate the effects of a single oral dose of ascorbic acid (1000 mg) over anxiety and other emotional states, as measured by STAI and VAMS, in a non-clinical sample of young graduate students. STAI and WAMS were applied two hours after the ingestion of ascorbic acid, a time point ascorbic acid reaches maximal concentration in human plasma after oral administration (Carr et al. 2013), the anxiety scores remained at basal levels (pre-treatment levels). However, a secondary analysis suggested the effects of ascorbic acid were dependent on trait anxiety (defined as low-to-moderate or high by a cut-off at the 75th percentile of the sample in the STAI T-anxiety scale). As compared to baseline, ascorbic acid produced a significant anxiolytic effect, which was restricted to subjects with high trait anxiety, as evidenced by a significant decrease in the STAI S-anxiety scale, and in the VAMS anxiety subscale scores.

Overall, placebo and ascorbic acid groups presented similar sociodemographic characteristics, and baseline trait and state anxiety and other emotional states, and depressive and anxious symptoms, ruling out the possibility that the observed effects are attributable to pre-treatment differences in these variables. Subjects with low-to-moderate trait anxiety receiving placebo were younger than those who received ascorbic acid, but a median difference of about two years in young adults seems unlikely to lead to differences in metabolism of ascorbic acid and cognition.

A limitation of the present study is the use of a sample with specific sociodemographic characteristics, composed primarily of young female university students undertaking post-graduation courses. This may reduce the external validity of our findings to other populations, especially males. Gender differences have been described in manifestations of trait and state anxiety (McLean and Anderson 2009), as well as in ascorbic acid metabolism (Blanchard 1991, Oreopoulos et al. 1993). Another limitation is the relatively small sample size in the secondary analysis of subjects with high trait anxiety. Therefore, replication in larger samples may be necessary to confirm, or not, our findings. In addition, variables such as regular diet and physical activity, and psychiatric diagnosis, were not controlled. However, the absence of differences in depressive and anxious symptoms in HADS, as well as the low rate of self-reported use of psychotropic medication, minimize these issues. The known anti-inflammatory effects of ascorbic acid (Sorice et al. 2014) can be a confounding variable in decreasing scores in STAI scale, however ascorbic acid supplementation may not alter

basal levels of inflammatory cytokines (Ellulu et al. 2015, Veskoukis et al. 2016). Despite these limitations, to our knowledge, this is the first report showing the ability of a single dose of ascorbic acid to elicit anxiolytic effects in humans. Future research on ascorbic acid would benefit from physiological measures of stress such as hemodynamic parameters, cortisol and oxidative stress.

STAI and WAMS were applied two hours after the ingestion of ascorbic acid, a time point ascorbic acid reached maximal concentration in human plasma after an oral administration (Carr et al. 2013). Daily supplementation with 500 or 1000 mg ascorbic acid maintain elevated plasma ascorbic acid levels for several hours (Mason et al. 2014, de Oliveira et al. 2015), which is a desired characteristic of a therapeutic drug.

Interestingly, our results are in line with the limited available data on humans. In a recent double-blind, randomized, placebo-controlled study (de Oliveira et al. 2015), lower levels of anxiety were found as measured by the Beck Anxiety Inventory, after oral supplementation with ascorbic acid 500 mg, daily for 14 days, in a non-clinical sample of high school students. A randomized single blind placebo-controlled trial also found decreased anxiety scores in the Depression Anxiety Stress Scale-21 in adults with type 2 diabetes, after oral supplementation with ascorbic acid (1000 mg daily for six weeks) (Mazloom et al. 2013). Animal studies also support the idea of an anxiolytic effect of ascorbic acid. Zebrafish exposed to methylmercury presented anxiety-like behavior, which was abolished by a pre-treatment with ascorbic acid (Puty et al. 2014). In another study (Hughes et al. 2011), anxiety-like behavior was decreased in rats fed with ascorbic acid for 3 months.

In our study ascorbic acid had effects only in subjects with high trait anxiety. Trait anxiety can be broadly defined as a predisposition to experience anxiety as a response to stress and psychological threats (Endler and Kocovski 2001, Reiss 1997).

The specific neurobiology of a given psychiatric disorder can be related to different patterns of outcomes. The idea of defining more homogeneous subgroups of patients, potentially able to respond differently to treatments, is one of the aims of the Research Domain Criteria (RDoC) initiative of the National Institute of Mental Health (Insel 2014). RDoC seeks to integrate neuroscience and psychiatry at research level through a classification with a strong biological validity (Cuthbert and Insel 2013). Although trait anxiety is a psychological concept and not a neurobiological marker, it has been already shown to affect cognitive and social responses and to be linked to the activation of brain circuits involving mainly the amygdala and prefrontal cortex (Bishop 2009, Etkin et al. 2004, Xu et al. 2013). In fact, the STAI is list-

ed in the RDoC matrix as a self-report measure of the construct acute threat/fear in the domain of negative valence systems. Since RDoC is strongly translational, it reflects the classical ethological differentiation between fear and anxiety (Tovote et al. 2015). The role of this differentiation remains to be understood at clinical level (Perusini and Fanselow 2015).

As previously mentioned, anxiety and depressive symptoms are frequently concomitant. They also seem to share several common neurobiological mechanisms (Goodwin 2015, Kessler et al. 2003). Studies in humans have shown that ascorbic acid also has antidepressant effects. In a double-blind, randomized, placebo-controlled trial (Brody 2002), a high dose of oral ascorbic acid (3000 mg), when administered for 14 days to healthy young adults, decreased scores in the Beck Depression Inventory, and increased sexual intercourse frequency. A double-blind, randomized, placebo-controlled study showed that the administration of 1000 mg of oral ascorbic acid for six months, adjunctive to fluoxetine in a pediatric sample, produced an additional decrease on depressive symptoms as compared to fluoxetine alone (Amr et al. 2013), suggesting that ascorbic acid may be useful for depressive pediatric patients (Lopresti 2015). Other studies have shown similar results in humans (Gautam et al. 2012, Khajehnasiri et al. 2013, Zhang et al. 2011). Our group has demonstrated antidepressant effects of ascorbic acid in mice subjected to tail suspension test (Binfaré et al. 2009), acute restraint stress (Moretti et al. 2013), chronic unpredictable stress, and on the depressive-like behavior induced by administration of the pro-inflammatory cytokine tumor necrosis-alpha (Moretti et al. 2012a, 2012b, 2015).

It is remarkable that pregabalin has been pointed as a first line choice for the treatment of generalized anxiety disorders (Baldwin et al. 2015, Martinotti et al. 2013). Interestingly, pregabalin present a rapid anxiolytic effect (Nutt et al. 2009), similar to the effect presented in this work. While pregabalin can decrease the release of glutamate (Martinotti et al. 2013), ascorbic acid can impair the glutamate binding to the NMDA receptor (Majewska et al. 1990). Furthermore, pregabalin can activate glutamate transport, clearing glutamate (Ryu et al. 2012), possibly increasing ascorbic acid release, as proposed earlier by the action of anionic high affinity glutamate transporters (EAAT2 e EAAT3) (Rebec and Pierce 1994). Based on these data, it is tempting to speculate that the anxiolytic effect of ascorbic acid can be related to a negative modulation of glutamatergic neurotransmission.

This work presents the first evidence for a rapid anxiolytic effect of ascorbic acid in a sub-sample of high trait anxiety individuals. The present results combined

with previous evidence suggesting that ascorbic acid may play a significant role in mood regulation, and the fact that its use as a nutritional supplement is safe and inexpensive (Verhamme et al. 2009), warrant further clinical studies dealing with its potential as an anxiolytic agent. Future mechanistic studies are necessary to provide clues for the development of new treatments. If confirmed, the use of ascorbic acid may be considered for the clinical practice, either as a supplement for normal individuals presenting high trait anxiety, or as an adjuvant for the treatment of anxiety disorders.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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