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Prolonged treatment with vitamins C and E separately and together decreases anxiety-related open-field behavior and acoustic startle in hooded rats

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ABSTRACT

Adult male and female hooded rats (about 110 days old) consumed vitamins C and E separately and combined together in their drinking water and were assessed for anxiety approximately 50 and then 80 days later in an open field and an acoustic startle apparatus. They were tested when 160+ days old, and then again at 190+ days. For both testing ages combined, the vitamins and their combination increased open-field ambulation and occupancy of the four center squares of the apparatus, while also accordingly decreasing occupancy of the four corners. Treatment with vitamins C and E separately and combined together also decreased acoustic startle amplitude. While there were several significant overall sex and testing age differences, there was no evidence that the vitamin treatment effects were dependent on the operation of either variable. There was also no evidence of synergism between vitamins C and E in their effects. It was suggested that decreases in anxiety produced by the vitamins may have arisen from their antioxidant properties, attenuation of cortisol activity or some as yet undetermined effects on anxiety-related brain structures and neurotransmitters.

1. Introduction

In recent years, there has been increasing interest in the behaviorally beneficial effects of the antioxidant vitamins C and E. This has largely arisen from evidence suggesting that they may slow down age-related degeneration of the CNS and associated cognitive declines especially in the presence of neurodegenerative diseases (Cantuti-Castelvetri et al., 2000; Martin et al., 2002; Morris et al., 1998, 2002). There is a reasonable amount of evidence from animal research of improved learning and memory following treatment with vitamin C (e.g., Arzi et al., 2004; Harrison et al., 2009a,b; Hasanein and Shahidi, 2010), although vitamin E may be less effective (Sumien et al., 2004) unless combined with vitamin C (Hasanein and Shahidi, 2010). Both vitamins have also been implicated in anxiety and psychological stress-related behavior in subjects not necessarily manifesting neurocognitive deficits. For example, 14 days of treating healthy young adults with vitamin C resulted in lower blood pressure, faster cortisol recovery and less state anxiety in response to a psychologically stressful experience (Brody et al., 2002). Pretreatment with vitamin C has also been shown to affect indices of fear in poultry such as attenuated tonic immobility and decreased neophobia in Japanese quail (Jones et al., 1996; Satterlee et al., 1993) and broiler chickens (Satterlee et al., 1993, 1994). Vitamin C was reported to reduce the time spent by mice in the dark versus aversive light compartment (De Angelis and Furlan, 1995) of a type of apparatus normally used for assessing anxiety (Harrison and May, 2009). Vitamin E deficiency has been associated with higher anxiety in mice (Desrumaux et al., 2004; Gohil et al., 2003) while dietary treatment with the vitamin may have reduced stress-related cardiac symptoms in pigs (Peeters et al., 2004). However, in one study, 20 mg/kg vitamin E (administration route not specified) has been shown to have no effect on anxiety in senescence-accelerated OXYS rats, but to increase anxiety in normal Wistar rats (Kolosova et al., 2006). It was therefore recently suggested (Hughes and Collins, 2010) that the failure for environmental enrichment to decrease anxiety in aging PVG/C rats when concurrently provided with vitamin E in their drinking water might have been due to interference with any anxiolytic effects of enrichment by the vitamin's possible pro-oxidant effects at higher doses (Rietjens et al., 2002). The present study was designed to assess the subsequent effects on anxiety-related behavior in adult rats of prolonged treatment with vitamins C and E (in their drinking water) individually and in combination. On the basis of previous research involving much shorter periods of treatment (Jones et al., 1996; Satterlee et al., 1989, 1993, 1994), it was anticipated that vitamin C would decrease anxiety. However, since a comparable level of vitamin E to that chosen for this study failed to have any effect on anxiety-related behavior in aging rats but appeared to interfere with enrichment-related anxiolysis (Hughes and Collins, 2010), it was expected that this vitamin on its own would either have no effect or even increase anxiety as shown earlier (Kolosova et al., 2006) because of its possible pro-oxidant properties (Rietjens et al., 2002). It should also be noted that vitamin E has been reported to have no effect on

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cognitive and psychomotor behavior in aged mice (Sumien et al., 2004). Because vitamin C can prevent vitamin E's pro-oxidant effects (Brigelius-Flohé and Traber, 1999), it was felt that treatment with both vitamins together might attenuate any anxiogenic influence of E. Although there does not appear to have been any investigations of the effects of a combination of vitamins C and E on anxiety-related behavior, in a recent study of treatment with each vitamin alone and together, there was improved learning and retention in normal rats following 30 days of oral C and C plus E, but not with E alone (Hasanein and Shahidi, 2010). However, diabetic rats improved with all three types of treatment as has also been shown for normal aged (but not for young) mice (Arzi et al., 2004).

Because of some differences in the way males and females were affected earlier (Hughes and Collins, 2010), the use of male animals only in previous studies of combinations of vitamins C and E (Arzi et al., 2004; Hasanein and Shahidi, 2010) and the need to consider both sexes in bio-behavioral research (Hughes, 2007; Zucker and Beery, 2010), rats of each sex were included in the present study.

2. Methods

2.1. Subjects

The subjects comprised 40 male and 40 female PVG/C hooded rats approximately 110 days old at the beginning of vitamin treatment. This strain was the same as that used in the earlier study of vitamin E effects (Hughes and Collins, 2010). The rats were housed in same-sexed pairs in 560×350×215-mm (length × width × height) cages. Each cage was divided in half by a 215-mm-high wire mesh partition that physically separated the two occupants but enabled them to see, smell and hear each other thereby minimizing any deleterious effects of social isolation. All rats were provided with their own drinking bottle and ad libitum food, and were kept in 12-h light: 12-h dark conditions with an ambient temperature of 20±1 °C.

2.2. Vitamin treatment procedure

For a period of 9 consecutive days prior to vitamin treatment, the rats' daily water intake and body weights were recorded. This information was then used for determining quantities of vitamin C (ascorbic acid) and vitamin E (DL-α-tocopherol acetate, Merck Chemicals Ltd) that needed to be added to their drinking water to achieve approximate daily doses of 75–100 mg/kg and 150–200 mg/kg respectively, when consumed separately or together. Ascorbic acid was added in the form of a soluble powder, and tocopherol as a water immiscible liquid containing 500 mg/ml. As males drank relatively less water per 100 g body weight prior to treatment (mean ± S.E.M. = 9.12±0.16 ml) than females (13.55±0.31 ml), the amounts of each vitamin added to the rats' drinking water differed for each sex i.e., for males and females respectively; 1.06 mg/ml and 0.76 mg/ml ascorbic acid, and 2.25 mg/ml and 1.45 mg/ml tocopherol.

The rats were provided with unadulterated drinking water (Control, $n=20$), or water containing vitamin C (Vit C, $n=20$), vitamin E (Vit E, $n=20$) or both (Vit C + E, $n=20$) for a total period of about 3 months. There were equal numbers of males and females in all treatment groups. For the duration of the study, amounts of fluid drunk by each rat were measured every 2 or 3 days when the drinking bottles were topped up. Once a week, all bottles were replaced with sterilized ones containing freshly prepared fluids.

2.3. Behavioral testing apparatus

The apparatus used to assess any behavioral effects of the vitamin treatment were a square open field and an acoustic startle chamber. The 600-mm×600-mm open field comprised a black Perspex floor divided into 16 numbered squares by means of a grid of white intersecting lines, and 300-mm-high clear Perspex walls. It sat on a 700-mm high table and was illuminated by dim fluorescent lighting (47 lx). The acoustic startle chamber consisted of one of a set of four (Med Associates, Fairfield, VT, USA) kept in a sound-attenuating room. Each chamber measured 600-mm×340-mm×560-mm and was constructed from sound-attenuating melamine. Inside the chamber were a speaker and a holding cage built from stainless steel rods. The walls and lid of the cage comprised horizontal rods 25 mm in diameter, and the floor comprised rods 45 mm in diameter. All rods were spaced 15 mm apart. Startle amplitudes were measured by Med Associates software in response to white noise bursts produced by a programmable audio generator. For this purpose, the holding cage was mounted on a load cell-based startle platform (250×115×45 mm) that enabled movement amplitudes to be amplified, digitized and recorded.

2.4. General procedure

After 52 days of treatment when the rats were approximately 160 days old, they all experienced a 5-min trial in the open field and a single session in the startle chamber. The order of tests varied for individual animals. Vitamin treatment was continued for another 31 days until the rats were about 190 days old when they experienced the same tests again. Following this second round of tests, the rats were weighed.

2.4.1. Open-field testing

Each rat was placed in the center of the apparatus and observed for exactly 5 min. Every 3rd sec (indicated by an auditory signal), the square it was occupying and if it was rearing up on its hind legs (unsupported or against a wall) or grooming were manually recorded on a prepared data sheet. It was then returned to a holding cage, fecal boluses it left removed and counted, and the apparatus washed with a disinfectant solution. Square occupancy data enabled the calculation of a measure of ambulation (or distance travelled) by counting the number of times a rat was seen in a different square from that occupied on the occurrence of an immediately preceding 3-sec auditory signal (Hughes and Beveridge, 1987). These data also provided information about the number of times the rat was seen occupying one of the four center squares or corners of the apparatus. Lower levels of ambulation, rearing and center squares occupancy, and higher levels of grooming, corner occupancy and defecation are regarded as indicative of higher anxiety in rats (Archer, 1973; Belzung, 1999).

2.4.2. Acoustic startle testing

Each rat was placed in a startle chamber and 3 min later subjected to 30 white noise bursts of 100 ms each with a rise–decay time of 10 ms, and a sound intensity of 95 dB. The data of the first 10 noise bursts were discarded to reduce the overall variance. The interval between each burst was 30 s. The magnitude or amplitude of each startle response was effectively determined from the amount of displacement of the startle platform. For each rat, the average movement amplitude in response to the 20 noise bursts comprised its startle score. Exaggerated startle responses are characteristic of heightened anxiety in both humans and animals (Rosen and Schulkin, 1998).

2.5. Statistical analyses

Body weights recorded at the end of the second round of testing and fluid drunk from the beginning until the end of treatment were subjected to separate 4 (vitamin treatment) × 2 (sex) ANOVAs. Average doses consumed during the course of the study of Vit C (alone and with Vit E) and Vit E (alone and with Vit C) were analyzed by separate 2 (alone and with the other vitamin) × 2 (sex) ANOVAs.

All behavioral responses were subjected to separate 4 (vitamin treatment) × 2 (sex) × 2 (testing age) repeated measures ANOVAs. Significant vitamin treatment effects were followed by post hoc pair-wise comparisons with Fischer PLSD tests ($P<0.05$). In order to determine the extent to which occupancy of the center squares and corners of the open field may have been dependent on the rats' levels of ambulation, the three measures were inter-correlated. To assess possible treatment effects on habituation, percent declines in open-field ambulation and rearing, and startle amplitude from the first (at 160+ days of age) to the second test (at 190+ days) were calculated and subjected to vitamin treatment × sex ANOVAs.

3. Results

3.1. Bodyweights, fluid intake and doses

Mean ± S.E.M body weights (g) of the rats in the Control, Vit C, Vit E and Vit C + E groups respectively were 274.30 ± 18.35, 278.92 ± 19.88, 278.95 ± 19.33 and 279.25 ± 19.07. The treatment effect was not significant [$F(3,72)=0.35$, $P>0.7$]. However, not surprisingly, the male rats were significantly heavier (mean ± S.E.M = 359.60 ± 3.67) than the females [196.11 ± 1.42, $F(1,72)=1644.69$, $P<0.00001$].

Fluid intake (ml/100 g body weight) for each treatment group respectively was 9.57 ± 0.55, 9.22 ± 0.56, 9.37 ± 0.56, 9.28 ± 0.52. While this treatment effect was not significant [$F(3,72)=1.69$, $P>0.1$], the sex effect was [$F(1,72)=1614.40$, $P<0.00001$]. Female rats drank significantly more fluid in proportion to body weight (11.71 ± 0.09) than males (7.01 ± 0.08).

The average dose of Vit C (mg/kg/day) consumed alone was 80.40 ± 2.04 and with Vit E, 81.21 ± 1.73. This difference was not significant [$F(1,36)=0.34$, $P>0.5$]. Comparable results for Vit E consumption were: alone = 165.17 ± 2.52, with Vit C = 169.27 ± 3.74. This difference was also not significant [$F(1,36)=2.85$, $P>0.1$]. For both vitamins, male rats consumed significantly lower doses than females i.e., Vit C, males = 73.74 ± 1.24, females = 87.87 ± 0.64, $F(1,36)=101.87$, $P<0.0001$; Vit E, males = 157.72 ± 2.16, females = 176.73 ± 2.82, $F(1,36)=35.37$, $P<0.0001$.

3.2. Behavioral results

Since grooming and defecation in the open field occurred so rarely in any treatment group, they were not considered further. Because of equipment failure with one of the startle chambers, the acoustic startle data of three Control (one male, two females) and four Vit C rats (three males, one female) had to be excluded from analyses. The effects of vitamin treatment on the behavioral measures for males and females recorded at each of the two testing ages are outlined in Table 1. The table also contains percent declines from the first to the second testing age for open-field ambulation and rearing, and acoustic startle amplitude.

Significant vitamin treatment effects occurred for open-field ambulation [$F(3,72)=3.23$, $P<0.03$], occupancy of center squares [$F(3,72)=5.32$, $P<0.002$] and occupancy of corners [$F(3,72)=9.11$, $P<0.0001$], but not for rearing [$F(3,72)=0.77$, $P>0.5$]. There was also a significant treatment effect for acoustic startle [$F(3,65)=3.12$, $p<0.035$], but not for percent decline in ambulation [$F(3,72)=2.30$, $P>0.08$], rearing [$F(3,72)=0.32$, $P>0.8$] or startle amplitude [$F(3,65)=1.66$, $P>0.1$] . In the absence of any significant interactions between treatment, sex and testing age for any measure, the significant main effects of vitamin treatment for both sexes and both treatment ages combined are outlined in Figs. 1 and 2.

While significantly more open-field ambulation occurred with each of the three treatment groups than with control rats (see Fig. 1A), there were no significant differences between the vitamin groups. Male rats displayed significantly less ambulation (mean ± S.E.M = 27.56 ± 1.50) than females [37.79 ± 1.33, $F(1,72)=27.83$, $P<0.0001$], and all rats engaged in significantly more of the behavior at 160+ days (39.20 ± 1.41) than at 190+ days [26.15 ± 1.49, $F(1,72)=59.51$, $P<0.0001$]. Although the sex difference for rearing was not significant [males = 17.59 ± 1.20, females = 18.50 ± 0.95, $F(1,72)=0.35$, $P>0.5$], significantly more of the behavior occurred at 160+ days (21.36 ± 0.89) than at 190+ days [14.73 ± 1.08, $F(1,72)=27.89$, $P<0.0001$]. The sex difference for percent decline was significant for ambulation [males = 42.72 ± 5.60, females = 22.69 ± 5.59, $F(1,72)=6.86$, $P<0.015$], but not for rearing [males = 15.24 ± 12.38, females = 21.17 ± 8.43, $F(1,72)=0.16$, $P>0.6$].

Rats that were treated with each vitamin alone or in combination were seen in the four center squares significantly more often than control animals (Fig. 1B), but there were no significant differences between the three vitamin groups. However, there was no significant sex [males = 5.62 ± 0.44, females = 6.64 ± 0.50, $F(1,72)=2.85$, $P>0.09$] or testing age effect for this measure [160+ days = 6.57 ± 0.39, 190+ days = 5.69 ± 0.48, $F(1,72)=2.43$, $P>0.1$]. The significant vitamin treatment effect on occupancy of the four corners accordingly arose from less corner occupancy shown by each of the treatment groups (Fig. 2A), but there were no significant effects of either sex [males = 66.62 ± 1.90, females = 64.85 ± 1.66, $F(1,72)=0.64$, $P>0.4$] or testing age [160+ days = 64.25 ± 1.19, 190+ days = 67.22 ± 1.83, $F(1,72)=2.84$, $P>0.09$].

While occupancy of the corners of the open field was significantly negatively correlated with both ambulation [$r(78)=-0.45$, $P<0.001$]

Table 1
Mean (± S.E.M.) behavioral measures for male and female rats in each vitamin treatment group recorded at 160+ (Test 1) and 190+ days of age (Test 2). See text for ANOVA results.

	Control		Vit C		Vit E		Vit C + E	
	M	F	M	F	M	F	M	F
Testing at 160+ days of age (Test 1):								
Ambulation	33.60(4.42)	41.30(3.52)	36.20(3.64)	44.70(4.16)	36.20(3.49)	46.50(3.45)	36.00(4.09)	39.10(3.56)
Rearing	21.90(2.17)	16.80(1.87)	22.50(3.25)	23.40(1.29)	21.10(3.66)	24.70(2.72)	19.60(2.96)	20.90(1.22)
Center squares occupancy	4.70(0.79)	5.60(1.33)	6.70(1.23)	7.50(0.95)	6.20(0.66)	8.10(1.02)	5.30(0.92)	8.50(1.50)
Corner occupancy	69.20(2.06)	70.80(3.17)	59.80(4.65)	63.60(2.68)	61.10(3.54)	59.10(3.58)	66.00(2.98)	64.40(2.92)
Startle amplitude	392.90(45.77)	422.89(67.79)	230.23(38.86)	232.10(51.34)	273.46(46.95)	213.55(34.09	389.21(60.79)	234.78(37.10)
Testing at 190+ days of age (Test 2):								
Ambulation	14.20(2.39)	20.80(1.98)	24.30(4.43)	33.30(2.61)	21.00(3.05)	34.90(4.63)	19.00(2.96)	41.70(3.48)
Tests 1 to 2 % decline in ambulation	54.03(9.58)	47.06(5.17)	28.92(15.96)	18.30(10.69)	36.97(9.97)	19.91(13.89)	50.95(7.14)	5.48(10.25)
Rearing	11.50(3.05)	14.10(3.05)	14.60(2.40)	12.90(2.25)	16.80(3.58)	13.80(2.06)	12.70(3.32	21.40(4.18)
Tests 1 to 2 % decline in rearing	34.39(19.57)	7.92(17.94)	15.74(31.57)	40.82(13.16)	−12.87(25.66)	42.45(8.99)	23.70(21.99)	−6.52(21.37)
Center squares occupancy	3.40(0.83)	2.70(0.88)	7.40(1.07)	4.70(1.26)	6.80(1.79)	7.70(1.15)	4.50(0.96)	8.30(1.71)
Corners occupancy	82.10(2.61)	78.00(3.47)	66.30(5.50)	65.30(4.17)	59.00(7.30)	57.00(5.22)	69.50(3.38)	60.80(3.68)
Startle amplitude	321.24(58.98)	229.92(32.02)	179.69(39.97)	250.07(54.34)	271.37(62.30)	164.02(31.89)	297.47(76.22)	139.91(25.65)
Tests 1 to 2 % decline in startle	12.30(11.54)	26.70(13.21)	13.67(14.75)	−23.34(31.70)	−6.28(20.71)	17.58(15.94)	29.73(8.65)	29.95(16.62)

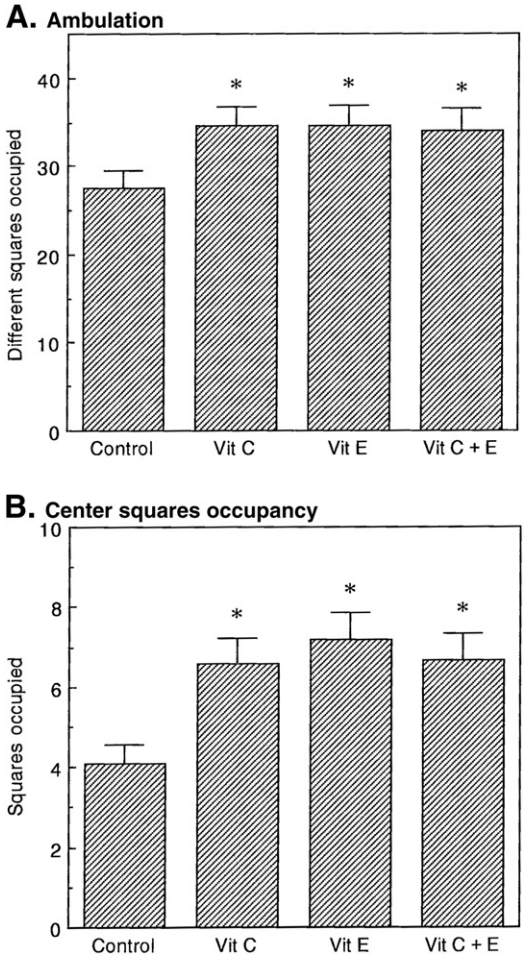

Fig. 1. Mean ± S.E.M. (A) open-field ambulation and (B) occupation of center squares following treatment with vitamins C and E separately, and together. *Significantly different ($P<0.05$) from control group.

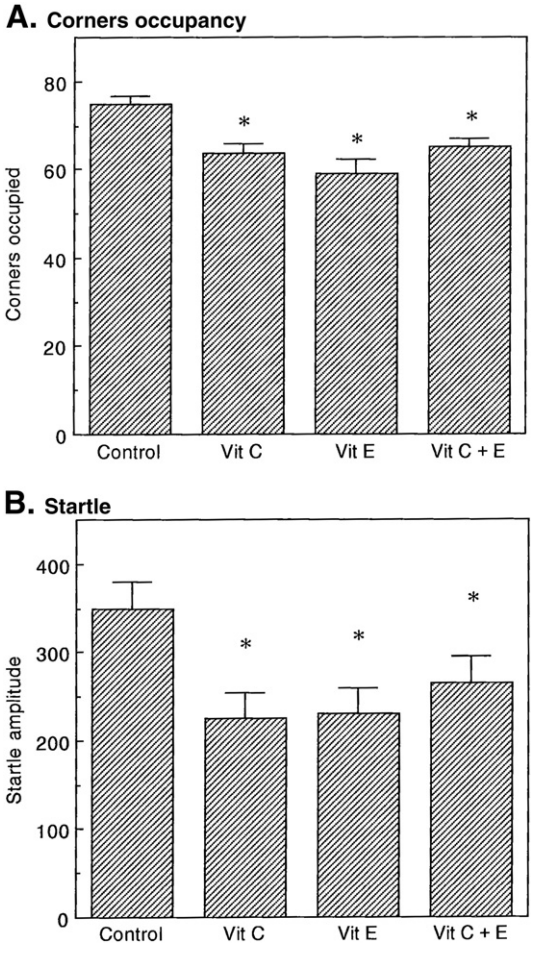

Fig. 2. Mean ± S.E.M. (A) occupancy of open-field corners and (B) acoustic startle amplitude following treatment with vitamins C and E separately, and together. *Significantly different ($P<0.05$) from control group.

and occupancy of the center squares [$r(78) = -0.57$, $P<0.001$] for all rats combined, the correlation between ambulation and occupancy of the center squares was not significant [$r(78) = 0.21$, $P>0.05$].

The significant vitamin treatment effect for acoustic startle amplitude arose from significantly higher amplitudes shown by control rats than by those in any vitamin group (Fig. 2B). There were no significant differences between the three treatment groups. The difference in this response between males (302.60 ± 26.61) and females (229.23 ± 19.24) was not significant [$F(1,65) = 3.33$, $P>0.07$], but for all rats combined the amplitudes were significantly higher at 160+ days (297.26 ± 18.84) than at 190+ days [235.57 ± 19.02, $F(1,65) = 15.18$, $P<0.0002$]. The sex difference in percent decline between the two testing ages was not significant [males = 13.19 ± 7.34, females = 14.49 ± 10.64, $F(1,65) = 0.03$, $P>0.8$].

4. Discussion

The lack of any effect of the vitamin treatment on the rats' bodyweights and their fluid intake suggests that their growth had been neither retarded nor enhanced by their experience, and that the vitamin solutions were no less palatable than unadulterated water. The higher volume of fluid drunk in proportion to body weight shown by females is consistent with earlier observations (Hughes and Collins, 2010) and has been ascribed to more diuretic activity than males because of females' lower levels of circulating antidiuresis-related hormones (McGivern et al., 1996; Wang et al., 1980). The higher female volume is probably why this sex consumed slightly higher doses of both vitamins than males. However, as none of the behavioral results were dependent on the sex of the rats, it is unlikely that the small differences in the amounts consumed by males and females were of any pharmacological significance. The doses achieved were well within the target range of both vitamins and were clearly effective in modifying the rats' behavior. It should be noted that the doses of vitamin E (165–169 mg/kg) were high for humans in terms of bodyweight, and in excess of the 400 IU/day that may be the lower limit for increasing all-cause mortality (Miller et al., 2005). However, this type of translation from rats to humans is inappropriate as it does not take into account the species differences in metabolic rate, blood volume, caloric expenditure, body size etc. If extrapolation of a rat dose to a human dose is carried out by normalizing body surface area (as recommended by the USA Food and Drug Administration), then a more realistic human equivalent dose can be achieved (Reagan-Shaw et al., 2007). In the case of vitamin E used in the present study, a rat dose within the range of 165–169 mg/kg translates to about 26.8–27.4 mg/kg for a human being. Besides, doses of 100–200 mg/kg vitamin E are not unusual in rat studies and have been shown by some authors to benefit cognitive behavior e.g., Comin et al., 2010; Socci et al., 1995; Yamada et al., 1999.

The results of the vitamin treatment revealed a similar pattern of behavioral effects in both types of apparatus. Overall, they were consistent with each vitamin having led to lower levels of anxiety whether consumed on its own or in combination with the other. This was suggested by increased treatment-related open-field ambulation and center squares occupancy (plus the inevitable opposite outcome for occupancy of corners), and decreased startle responding. However,

it is possible that some of the effects observed may not necessarily have been due to lowered anxiety but could have merely arisen from heightened activity. For example, in the open field, treatment-related increases and decreases of center squares (Fig. 1B) and corners (Fig. 2A) may have merely been a reflection of increased ambulation by vitamins C and E and their combination (Fig. 1A) that was not necessarily associated with reduced anxiety. While this was possible for corner occupancy (that was significantly negatively correlated with ambulation), it seems less likely for center squares occupancy because of this measure's lack of a significant correlation with ambulation. Further support for the open-field results having arisen from decreased anxiety was found in the anxiolytic-like effects of vitamins C and E separately and combined on acoustic startle amplitude, a response which is independent of locomotor activity (Kokkinidis and Anisman, 1978).

It is interesting that evidence of decreased anxiety with vitamin E on its own was contrary to what was described in an earlier report (Hughes and Collins, 2010). However, this may have been due to differences in ages of the rats at the commencement of treatment, namely 330 days in the earlier but 90 days in the present study. It is conceivable that any pro-oxidant (at the expense of antioxidant) properties of vitamin E were less likely to have affected the younger than the older animals (Poynter and Daynes, 1998). The fact that, for each measure, the combination of vitamins C and E was no more (or less) effective than either one separately was unexpected and at odds with a report of no effects of vitamin E on its own but significant improvements in learning and memory following chronic treatment with either vitamin C alone, or vitamins C and E combined (Hasanein and Shahidi, 2010). However, it has also been shown that the antioxidant potential of vitamins C and E administered together is no greater than for either vitamin on its own (Zaidi and Banu, 2004). The present investigation appears to be the first to show conclusive evidence of decreased anxiety-related behavior with chronic vitamin E. It may therefore be that long periods of treatment with vitamin E are required for any anxiolytic effect to become apparent. The results from both types of apparatus supported the expectation that vitamin C on its own would lead to lower anxiety, and are thus consistent with several earlier findings (Brody et al., 2002; De Angelis and Furlan, 1995; Jones et al., 1996; Satterlee et al., 1989, 1993, 1994).

The absence of any change in the effectiveness of the vitamin treatment from testing at day 160+ to 190+ suggests relatively long-term anxiolysis. However, it should be remembered that, following the first round of testing, the rats continued having access to their appropriate drinking fluids until after their second round. While it is assumed that the results were due to prolonged treatment, the design of the study did not allow for any distinction to be drawn between chronic and acute effects.

As vitamins C and E are both scavengers of free radicals, any possible neuroprotective effects and cognitive benefits in aging rodent and human populations are usually ascribed to their antioxidant properties whereby oxidative stress-related neural damage is reduced (Martin et al., 2002). It is possible that these antioxidant properties might also be responsible for the two vitamins' anxiolytic effects, because they have been shown to modulate immobilization-induced oxidative stress in the rat brain as indicated by increased activity of superoxide dismutase, glutathione-*S*-transferase and catalase, and decreased lipid peroxidation (Zaidi and Banu, 2004). Although in the present study no measurements were made of either increases in amounts of treatment-related brain ascorbate and tocopherol, or reductions in oxidative stress levels, from previous research such changes would have been expected (Rice, 2000; Vatassery et al., 1988; Zaidi and Banu, 2004). However, it is also possible that the vitamins' anxiolytic effects might have arisen from some influence other than their antioxidant properties alone, such as their ability to attenuate levels of the stress-related hormone, cortisol, in a range of species (Reddy et al., 1987; Satterlee et al., 1989; Shaheen et al., 1993; Webel et al., 1998). In addition, both vitamins have been implicated in brain structures and neurotransmitter systems that are known to be important for anxiety. For example, very high levels of ascorbate are found in structures associated with anxiety in both rats and humans, namely the amygdala and hippocampus (Mefford et al., 1981; Milby et al., 1982). Ascorbate may act as a neuromodulator of a number of transmitters and their associated behaviors (Harrison and May, 2009) including the inhibition of NMDA receptor activity (Majewska et al., 1990). This could lead to decreased anxiety since NMDA antagonism has been shown to be anxiolytic in rats and mice in a similar fashion to benzodiazepines (Plaznik et al., 1994). Vitamin E can enhance binding of the $GABA_A$ receptor (Takahashi et al., 1984) which could conceivably ameliorate anxiety since reduction of such binding has been reported to produce benzodiazepine-resistant anxiety in mice (Sibille et al., 2000). And both vitamins C and E may normalize brain serotonin (Lee et al., 2001) that has been implicated in some forms of human anxiety (Naughton et al., 2000) because of decreases in the state when treated with serotonin re-uptake inhibitors (Ballenger, 1999). Although involvement of one or more of these systems is a possibility, the mechanisms for effects of vitamins C and E on anxiety-related behavior remain to be determined.

The lower level of open-field ambulation observed in male than in female rats was consistent with many earlier reports of females being the more active of the two sexes (Archer, 1975). Although the rats' slightly older ages might account for the changes between testing at 160+ and 190+ days in open-field ambulation and rearing, and acoustic startle amplitudes, it seems more credible that their earlier experiences with the testing procedures were mainly responsible since they were only one month older for their second round of testing. In other words, the rats had most likely habituated to some extent to the experimental setting and procedure during their first tests. As such habituation can be viewed as an elementary form of learning and memory (Thiel et al., 1998), any cognitive-enhancing effects of the vitamin treatment might have been reflected in steeper declines between the first and second round of testing in open-field ambulation and rearing, and acoustic startle. As this clearly did not happen, it is possible that the particular procedures and treatment adopted in the present study were more specifically relevant to anxiety than to cognition. While the steeper decline in open-field ambulation for males was contrary to the view that female rats habituate to novelty more rapidly than males (Green et al., 1975; Hughes, 1990), it should be noted that a more rapid reduction in within-session exploration of novelty by males has also been reported (Archer, 1975). In addition, a lack of between-sessions habituation in open-field ambulation over 10 days of testing has been observed for adult females (Bronstein, 1973).

From the profile of effects of the vitamin C and E treatment on the particular anxiety-related responses recorded, it seems reasonable to conclude that separately and together they were anxiolytic. There was also no evidence of synergism of the two vitamins because together, they were no more effective than when experienced on their own. The results of the current study add to growing evidence that both vitamins can have significant benefits for behavior. While most such evidence to date relates to their cognitive benefits, the present findings support the likelihood that they may also have emotional benefits. However, given the very long period of treatment adopted in the present study, from a practical point of view it would be useful to determine if much shorter periods might also be beneficial, as has been shown with vitamin C for poultry (Jones et al., 1996; Satterlee et al., 1989, 1993, 1994).

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