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Grape powder treatment prevents anxiety-like behavior in a rat model of aging

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ABSTRACT

Earlier, we have reported that grape powder (GP) treatment prevented pharmacologic and psychological stress-induced anxiety-like behavior and memory impairment in rats. Protective effects of GP were attributed to its antioxidant effects. In this study, we tested the hypothesis that age-associated behavioral and cognitive deficits such as anxiety and memory impairment will be ameliorated with GP treatment. Using a National Institute of Aging recommended rodent model of aging, we examined a potentially protective role of antioxidant-rich GP in age-associated anxiety-like behavior and memory impairment. Male Fischer 344 rats were randomly assigned into 4 groups: young rats (3 months old) provided with tap water or with 15 g/L GP dissolved in tap water for 3 weeks, aged rats (21 months old) provided with tap water or with GP-treated tap water for 3 weeks (AG-GP). Anxiety-like behavior was significantly greater in aged rats compared with young rats, GP-treated young rats, or aged control rats ($P < .05$). Also, GP treatment prevented age-induced anxiety-like behavior in AG-GP rats ($P < .05$). Neither short-term nor long-term age-associated memory deficits improved with GP treatment in AG-GP rats. Furthermore, aged rats showed increased level of physiological stress (corticosterone) and increased oxidative stress in the plasma (8-isoprostane) as well as in selected brain areas (protein carbonylation). Grape powder treatment prevented age-induced increase in corticosterone levels and plasma 8-isoprostane levels in aged rats ($P < .05$), whereas protein carbonylation was recovered in the amygdala region only ($P < .05$). Grape powder by regulating oxidative stress ameliorates age-induced anxiety-like behavior in rats, whereas age-associated memory deficits seem unaffected with GP treatment.

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1. Introduction

It is well documented that aging is accompanied with decline in cognitive and emotional functions [1]. Several interventions

have been proposed over the years to promote healthy aging including moderate physical exercise and a balanced diet [2]. Many studies have suggested that polyphenolic compounds present in fruits and vegetables rich in color such as grapes help

Abbreviations: EPM, elevated plus maze; CTGC, California Table Grape Commission; GP, grape powder; OFT, open-field test; RAWM, radial arm water maze test.

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in coping with anxiety and improving memory and cognition during aging [3,4].

These health benefits are proposed to occur via the antioxidant and anti-inflammatory activities of fruits and vegetables [5]. In particular, a lot of attention has been focused on potential health benefits of grapes [6]. Several studies have reported beneficial effects of grapes on cardiovascular activities [7], as well as on mental well-being [3,4,8]. Antioxidant properties of grapes attributed predominantly to its numerous polyphenolic constituents including resveratrol are well characterized [9–13] and largely believed to be responsible for its beneficial effects. Multiple signaling pathways involving antioxidant [14,15], anti-inflammatory [16], and/or antiapoptotic [17] mechanisms are purported to enable the protective effect of grapes. Recently, using a pharmacologic model of oxidative stress [14], we established that California Table Grape Commission (CTGC) provided grape powder (GP) treatment ameliorated oxidative stress-induced anxiety-like behavior, memory impairment, and hypertension in rats [18]. This study has prompted us to further investigate the protective effects of this GP in a nonpharmacologic model and examine whether beneficial effects of GP are limited to pharmacologically induced models of oxidative stress or extend to other genetic models that are well known to be associated with oxidative stress [19].

It is well recognized that oxidative stress, which results when the production of reactive oxygen species overwhelms antioxidant defense system [1], is critical for aging. There is extensive evidence suggesting the involvement of oxidative stress in aging processes of the brain [1]. Relevant to this, oxidative stress has been implicated in Alzheimer disease, Parkinson disease, and several other age-related neurodegenerative illnesses [20]. Numerous epidemiologic studies have suggested that dietary supplementation with antioxidant-rich fruit or vegetable extracts might decrease the enhanced vulnerability to oxidative stress that occurs during aging leading to improvements in motor and cognitive behavior [21]. Therefore, in order to fully investigate the protective effects of grapes on age-related anxiety and memory deficits, it must be tested in an aging model. A National Institute of Aging recommended that rodent F344 model of aging seems a good fit for this study because this is known to have elevated oxidative stress and has an aged phenotype [22–24].

Effects of nutritional intervention including tea, fruit, and vegetable extracts on cognitive and motor function have been tested in the F344 model by others [25,26]. However, 2 of the most pronounced age-associated behaviors, that is, anxiety and cognition, have not been examined in this model. Protective effects of grapes on simultaneous occurrence of these behaviors also are not known. In the present study using the F344 rodent model of aging, we examined the role of antioxidant-rich GP, provided by the CTGC in age-associated anxiety-like behavior and memory impairment, and also examined the level of physiological stress and levels of oxidative stress systemically as well as in specific areas of the brain including the prefrontal cortex, hippocampus, and amygdala. These regions are implicated in regulation of anxiety [27] and cognition [28], amenable to nutritional intervention [18], and also regarded as oxidative stress-susceptible regions [29].

Finally, this study using the National Institute of Aging-recommended rodent F344 model of aging will test the hypothesis that age-associated behavioral and cognitive deficits such as anxiety and memory impairment are ameliorated with GP treatment.

2. Methods and materials

2.1. Freeze-dried GP

Freeze-dried GP was provided by the CTGC. The powder was received in small sealed plastic bags and stored at -80°C . Grape powder solution was prepared fresh daily as published previously [18] by dissolving the powder in tap water at a concentration of 15 g/L. This GP dose produced most pronounced effects on rat behavior as reported previously [27]. Detailed composition and purity of this powder have been described in Allam et al [18].

2.2. Animals

All experiments were conducted in accordance with the National Institutes of Health guidelines using approved protocols from the University of Houston Animal Care Committee. Three-month-old young male Fischer 344 rats (250–275 g) and 21-month-old male Fischer 344 rats (400–450 g) were purchased from Charles River, Wilmington, MA, USA. These rats were housed with a 12-hour light, 12-hour dark cycle (lights on at 0600 hours) in a climate-controlled room with food and water provided ad libitum. After arrival at the animal research facility, all rats were allowed 1 week for acclimatization.

2.3. Experimental design

Male Fischer 344 rats were assigned into 4 groups (8–10 rats/group). (1) Young rats (Y-CON), (2) GP-treated young rats (Y-GP; provided with 15 g/L GP dissolved in tap water for 3 weeks), (3) aged control rats (AG-CON), and (4) GP-treated aged rats (AG-GP; provided with 15 g/L GP dissolved in tap water for 3 weeks). The Y-GP and AG-GP were pretreated with GP for 3 weeks prior to behavior testing and continued to receive GP-treated water until euthanized with decapitation. All rats were subjected to anxiety-like behavior tests followed by memory test. Upon conclusion of behavior and cognition tests, rats were killed by decapitation. Blood and brain tissues were collected, and corticosterones and indices of oxidative stress were measured as previously [14,15,30] (Fig. 1).

2.4. Anxiety-like behavior tests

First, elevated plus maze (EPM) was conducted followed by open-field tests (OFTs), as previously published [14,15].

2.4.1. Elevated plus maze

The less amount of time spent in open arms is considered as a measure of anxiety-like behavior. A standard rat EPM with 2 walled arms and 2 open arms extending 43 cm from a 10-cm central area (Med Associates Inc, St Albans, VT, USA) was used. The arms of the maze were approximately 90 cm above the floor. The rat's movements were tracked visually. Each session was

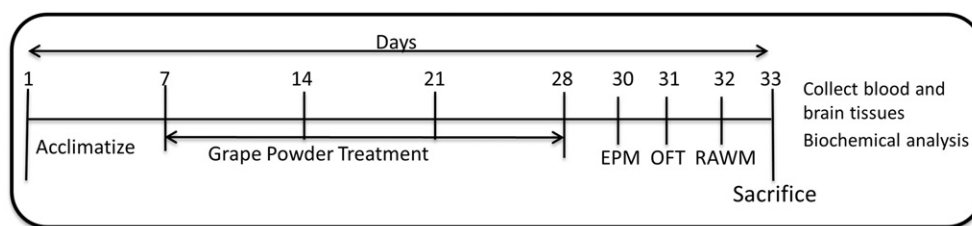


Fig. 1 – A schematic representation of the experimental regimen. Male Fischer 344 rats were acclimatized for 1 week and assigned into 4 group: (1) Y-CON, (2) Y-GP (provided with 15 g/L GP dissolved in tap water for 3 weeks), (3) AG-CON, (4) AG-GP (provided with 15 g/L GP dissolved in tap water for 3 weeks). Behavior tests were conducted 48 hours after the GP protocol completion.

started by placing the rat in the central area facing the open arms of the maze and lasted 5 minutes. In between each test animal, the maze was wiped down with alcohol. The amount of time the rat spent in the open arms was noted [31].

2.4.2. Open-field test

Rodents, in general, have tendency to explore new area. Normally, they spend an equal amount of time in the center and periphery. However, anxious rats spend more time toward the periphery and do not show preference to explore a new area. Rats were placed in the center of the open field (60 × 40 cm) and left free to explore the arena for 15 minutes, and movement was quantified using Opto-Varimax Micro Activity Meter v2.00 system (Optomax; Columbus Instruments, Columbus, OH, USA) as previously published [14,15]. Total activity, ambulatory activity, and distance covered were recorded.

2.5. Memory function test

The radial arm water maze (RAWM) procedures were done as previously published [18]. The RAWM consisted of a black circular pool filled with water at 25°C containing 6 swim paths in a dimly lit room. Each rat was randomly assigned a goal arm which contains a hidden black platform near the end of the arm. The rats were randomly released at an arm different from the goal arm and allowed to swim and locate the platform, which is submerged 1 cm under water. The rats were allowed 1 minute for each learning trial or memory test. An error was counted when the rat entered more than halfway into an arm other than the goal arm, or if the rat entered more than half of the goal arm but failed to approach the platform. Number of errors ranged from 1 to 7, as the rat can only swim into a total of 7 arms within 1 minute. If the rat failed to locate the platform within 1 minute, it was manually guided to the platform and scored with 7 errors. Upon reaching the platform, the rat was allowed for a 15-second rest before the next trial began. Each rat was subjected to a set of 6 learning trials (trials 1–6) followed by a 5-minute rest period, and then another set of 6 learning trials (trials 7–12). The short-term memory (STM) test was conducted 30 minutes after the end of 12th trial. This was followed by the long-term memory (LTM) test that was given 24 hours later.

2.6. Plasma corticosterone

Corticosterone level in plasma was measured using an enzyme immunoassay (EIA) kit (#500655; Cayman Chemical Company, Ann Arbor, MI, USA) as previously published [32].

2.7. Indices of oxidative stress

8-Isoprostane levels in serum were measured using an EIA kit (#516351; Cayman Chemical Company). Isoprostanes are a family of eicosanoids of nonenzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals [14]. The OxyBlot Protein Oxidation Detection Kit (#S7150; EMD Millipore Corp, Temecula, CA) was used for immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions. Equal amount (4 µg) of protein homogenate from different brain regions prepared using our published protocol [28] were subjected to this kit-based reaction following the manufacturer's instructions, which allows for detection of carbonylation of proteins in the homogenates using Western blotting method [29].

2.8. Brain dissections and preparation of homogenates

Rats were anesthetized using isoflurane anesthesia (57319-479-06; Phoenix Pharmaceuticals, Burlingame, CA, USA) 24 hours after the conclusion of all behavior tests. The brains were quickly removed and rapidly frozen at –80°C until analysis. The hippocampus, amygdala, and prefrontal cortex were identified according to Paxinos and Watson [33] and grossly dissected out and homogenized; protein concentration was determined as previously published [15].

2.9. Western blot analysis

Homogenates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting. The following dilutions were used for detection of specific proteins: c-fos (1:500 dilution; Cat. No. ab184666) was purchased from Abcam, Cambridge, MA, USA, and loading control β-actin (1:1000 dilution) and antimouse horseradish peroxidase–linked secondary antibody (1:1000) were used as needed. The intensity of each immunoreactive band on immunoblots (normalized to the β-actin loading control) was determined using Alpha Ease FC 4.0 (Alpha Innotech Corp, San Jose, CA, USA).

2.10. Statistical analyses

Data are expressed as means ± SEM. Significance was determined by 1-way analysis of variance and Tukey post hoc test (GraphPad Software, Inc, La Jolla, CA, USA). For corticosterone, 8-isoprostane, and Western blot analysis, significance was determined using 2-tailed t test (GraphPad Software, Inc). For these studies, with an

effect of 35%, variance of means of 0.2, a significance of level of .05, and a power of 0.8, about 10 animals will be needed per group. A *P* value less than .05 was considered significant.

3. Results

3.1. Anxiety-like behavior tests

Elevated plus maze and OFTs were conducted to test anxiety-like behavior in rats. Elevated plus maze model is based on rat's aversion for open spaces. This aversion leads to a behavior termed as *thigmotaxis*, which means avoidance of open areas by restricting movements to enclosed spaces or to the edges of a confined space. Increased time spent in the closed arms during a 5-minute session is indicative of high anxiety-like behavior. The EPM results suggest that Y-CON and Y-GP rats spent an equal amount of time in the open arms 207 and 211 seconds, respectively. The AG-CON rats spent a significantly reduced time (27 seconds) in the open arms when compared with Y-CON (207 seconds) rats, indicating an aging effect on this parameter. Furthermore, AG-GP rats spent more time in the open arms (66 seconds) as compared with the AG-CON (27 seconds), suggesting that GP improved anxiety-like behavior of the aged group ($F_{3,29} = 87.8$; $P < .05$) but not in young rats (Fig. 2 A).

In the OFT, it is observed that rodents typically spend equal time exploring the periphery of the arena as well as the unprotected center area of the open-field apparatus. Rats that spend significantly more time exploring the unprotected center area demonstrate reduced anxiety-like behavior. Total and ambulatory activity counts and the distance traveled for Y-CON and Y-GP were the same. In contrast, the AG-CON rats had significantly lower total ($F_{3,29} = 27.3$; $P < .05$; Fig. 2 B) and ambulatory ($F_{3,29} = 26.5$; $P < .05$; Fig. 2 C) activity counts and covered less distance ($F_{3,29} = 19.9$; $P < .05$; Fig. 2 D) than did the AG-CON rats, whereas AG-GP rats had higher activity counts and covered more distance than did the AG-CON rats, suggesting that GP improved anxiety-like behavior of the aged group.

3.2. Memory function test

Effect of GP treatment on memory function of young and aged rats was evaluated using RAWM test. Both Y-CON and Y-GP

rats made comparable errors in STM and LTM tests. Aged rats (AG-CON) made more errors than did young rats (Y-CON) in the STM ($F_{3,29} = 3.4$; $P < .05$) and LTM ($F_{3,29} = 4.1$; $P < .05$) analysis. Grape powder treatment did not significantly prevent AG-GP rats from making errors in the STM or LTM tests. Aged and GP-treated aged rats made comparable errors, whereas AG-CON made more errors than did Y-CON rats in the LTM analysis of the RAWM test (Fig. 3 A, B).

3.3. Plasma corticosterone levels

Plasma corticosterone is a systemic marker of physiological stress. The corticosterone levels were similar in the Y-CON and Y-GP rats. On the other hand, AG-CON rats had significantly higher levels when compared with Y-CON rats, indicating an aging effect on this parameter. Furthermore, AG-GP had lower corticosterone levels as compared with the AG-CON, suggesting that GP reduced the systemic stress of the aged group ($F_{3,24} = 7.4$; $P < .05$; Fig. 4).

3.4. Analysis of neuronal activation marker

An increased *c-fos* level is directly proportional to increased anxiety [34,35]. *c-fos* expression was significantly lower in the hippocampus and the amygdala in young rats when compared with the aged rats; only the amygdala region of the aged rats treated with GP showed reduced *c-fos* levels when compared with its age-matched controls, whereas GP showed no effect in the prefrontal cortex and hippocampus in both sets of rats (Fig. 5 A-C).

3.5. Analysis of oxidative stress markers

Plasma 8-isoprostane levels were comparable in both Y-CON and Y-GP rats. On the other hand, AG-CON rats exhibited significantly higher levels of 8-isoprostane when compared with Y-CON rats, indicating an aging effect. Furthermore, AG-GP rats had reduced 8-isoprostane level when compared with AG-CON rats, suggesting that GP alleviated the oxidative stress of the aged group ($F_{3,29} = 6.11$; $P < .05$; Fig. 6).

Another marker of oxidative stress, protein carbonylation was measured in the prefrontal cortex, hippocampus, and amygdala, brain areas considered susceptible to oxidative stress and previously reported to be important for anxiety-

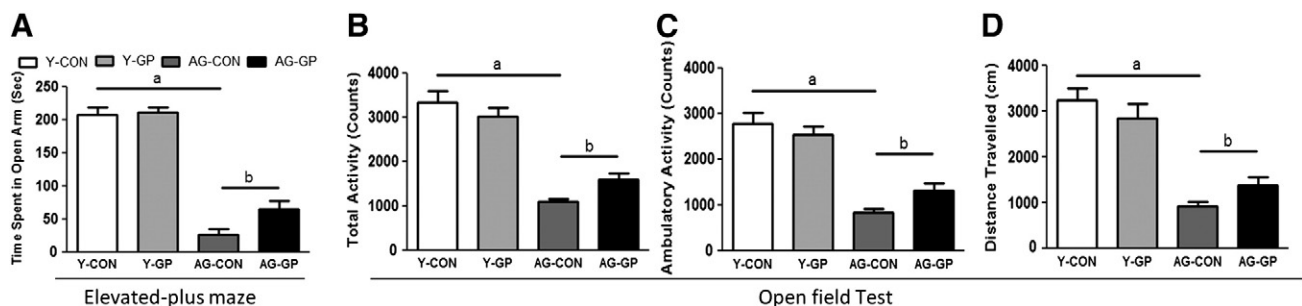


Fig. 2 – Examination of anxiety-like behavior in the EPM and OFT in the rats treated with/without GP. Elevated plus maze (A), OFT-determined total activity count (B), ambulatory activity count (C), and distance traveled (D). Means without a common letter differ ($P < .05$). Bars represent means \pm SEMs; $n = 8-10$ rats/group.

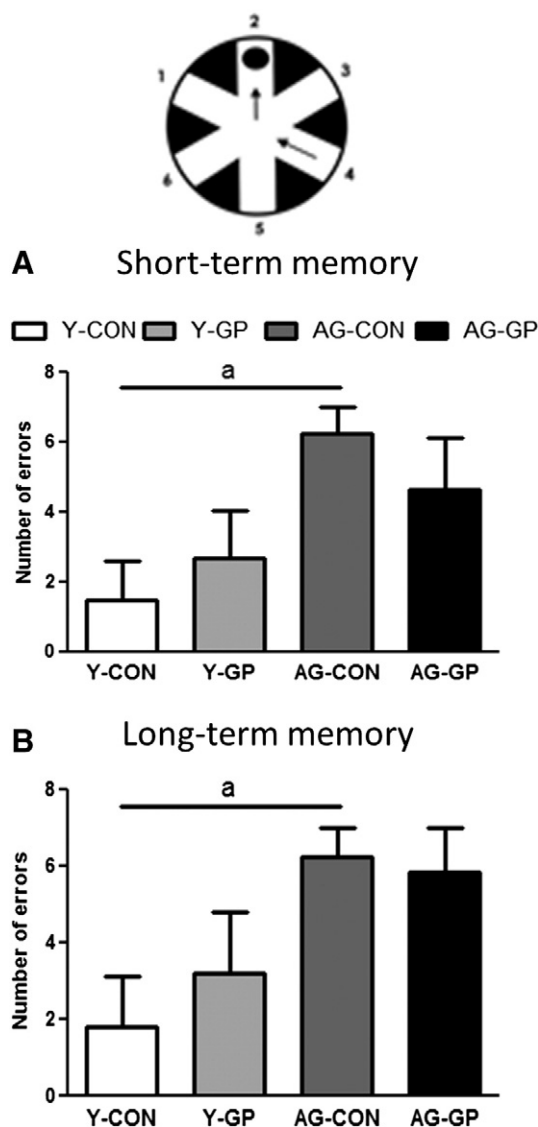


Fig. 3 – Examination of RAWM memory tests in rats treated with/without GP. Short-term memory (A) and LTM (B) were assessed by using a series of 12 RAWM trials. The RAWM apparatus is shown as an insert containing a circular water pool with 6 swim paths. Means without a common letter differ ($P < .05$). Bars represent means \pm SEMs; $n = 8-10$ rats/group.

like behaviors [28]. Protein carbonylation levels were not altered in the hippocampus but significantly decreased in the prefrontal cortex and amygdala of the young rats when compared with the aged rats. Surprisingly, GP only reduced the protein carbonylation in the amygdala of the aged rats when compared with its age-matched controls. Grape powder showed no effect in the prefrontal cortex and hippocampus of the aged rats (Fig. 7 A-C).

4. Discussion

Our laboratory in 3 separate studies has reported that CTGC freeze-dried GP prevented pharmacologically and psychologically induced anxiety and depression-like behaviors and also

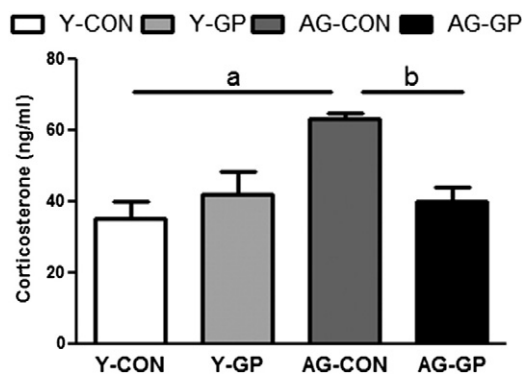


Fig. 4 – Examination of corticosterone levels in plasma. Corticosterone levels were measured using an EIA kit (Cayman). A and B, Means without a common letter differ ($P < .05$). Bars represent means \pm SEMs; $n = 6-8$ rats/group.

improved learning and memory function in rats [18,27,36]. In the present study, protective effect of grapes on age-related anxiety and memory deficits were examined using the F344 rat model of aging. These rats are reported have elevated oxidative stress with an aged phenotype [22–24].

Aging-induced anxiety-like behavior was prevented with GP treatment. This is in agreement with our previous studies in which we had reported antianxiety effect of GP in 3 separate rodent models [18,27,36]. Anxiety-like behaviors induced by L-buthionine-(S,R)-sulfoximine [18], single prolonged stress [27], and ovariectomy [28] were prevented with GP treatment. Interestingly, although STM and LTM deficits were observed in aged F344 rats, STM and LTM remained unaffected with GP treatment in this model, although in the previous 3 models, we had observed a nearly complete reversal of STM and LTM with GP treatment. Perhaps, age-associated memory deficits in aged F344 rats are irreversible and not amenable to nutritional intervention. Short-term memory and LTM reversals observed in the previous 3 models were all stress models and not genetic models. We also must point out that robust behavioral changes seem to occur in rats upon induction of pharmacologic [18], physiological [28], or psychological stress [27] rather than when genetically manipulated rodent models. The greater the stress, the bigger the change in behavior and the higher the response of GP.

Furthermore, our data suggest that antianxiety effects of GP are not model specific but condition selective and stress sensitive. The rationale for this consideration seems even more compelling considering our biochemical data. Glucocorticoid (corticosterone in rodents) is considered as a physiological marker of stress [37]. Our results suggested that aged F344 rats have increased plasma corticosterone levels, whereas GP treatment prevented an age-induced increase in corticosterones. This is in tandem with our previous observations in which behavioral and cognitive impairments noted in other rodent models [3,27,28] were associated with elevated plasma corticosterone levels [3,27,28]. In addition to examining the protective role of GP using systemic evaluation of stress, we also examined the level of neuronal stress using a biochemical marker of neuronal activation, c-fos [34,36]. We focused on 3 regions of the brain, namely, the hippocampus, amygdala, and prefrontal cortex, areas implicated in anxiety, cognition, and

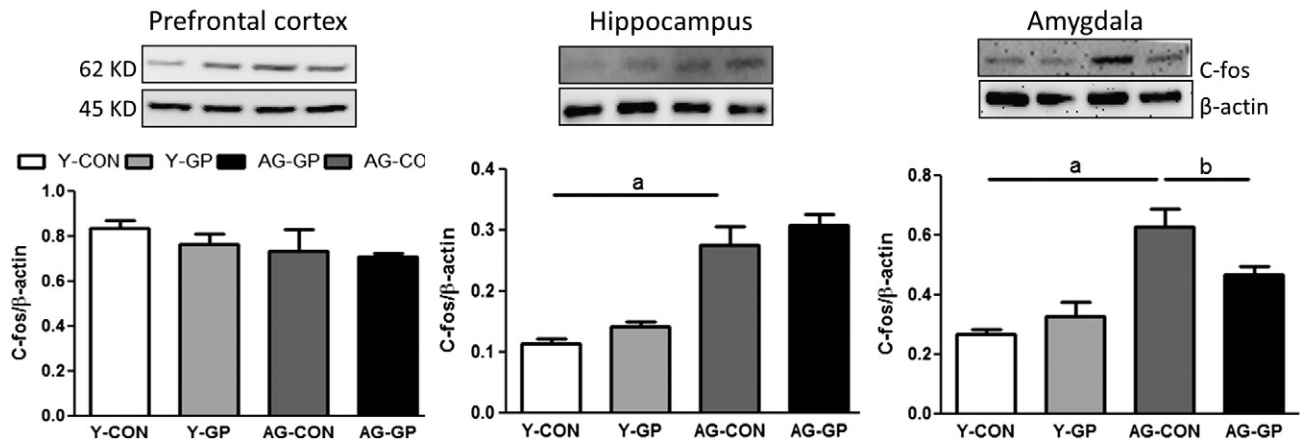


Fig. 5 – Examination of c-fos protein levels in the prefrontal cortex, hippocampus, and amygdala of rats treated with/without GP. Protein levels of c-fos were determined by Western blotting. A–C, The upper panels are representative blots for c-fos and the protein loading control β -actin. Bar graphs are ratios of c-fos to β -actin. Means without a common letter differ within the hippocampus, amygdala, and cortex ($P < .05$). Bars represent means \pm SEMs; $n = 8$ –10 rats/group.

psychological stress [27,29]. We observed an age-induced increase in c-fos levels in the hippocampus and the amygdala but not in the prefrontal cortex area of the brain. Furthermore, GP treatment significantly reduced the age-induced increase in c-fos expression in the amygdala but not the hippocampus region of the brain. Perhaps, mechanisms responsible for reversal of anxiety-like behavior in aged rats with GP treatment are localized within the amygdala. Amygdala is a well-recognized regulator of anxiety response [34,35]. Therefore, it is reasonable to expect this region to be altered with GP treatment.

Interestingly, these same brain areas also are considered to be highly susceptible to oxidative stress [27,29,36]. This is particularly relevant considering the protective effects of GP on aging-induced behavioral and cognitive deficits and the fact that the protective effects are most likely attributable to the antioxidant-rich nature of GP [38]. Several studies have suggested the role of antioxidant mechanisms in enabling neuroprotective effect of grapes [3,21,27,28,38]. Our previous studies have demonstrated GP-mediated prevention in stress-induced decline of antioxidant enzymes glutathione reductase-1 and glyoxalase-1 as well as that of brain-derived neurotrophic

factor, in specific regions of the brain including the hippocampus [18,28]. It is possible that a reduced pool of antioxidant enzymes contribute to the failing antioxidant defense system, which leads to an imbalance between cellular production of reactive oxygen species and the counteracting antioxidant mechanisms. Reduced antioxidant defense, together with diminished brain-derived neurotrophic factor levels, causes behavioral impairments. In addition, depletion in these proteins is prevented by GP treatment by regulating the expression of these proteins. Relevant to this, we observed that GP treatment prevented aging-induced rise in oxidative stress levels examined via assessing plasma 8-isoprostane levels in the serum as well as evaluation of oxidative stress in the brain using protein carbonylation.

It is clear from our data that aging-induced erroneous physiological and behavioral outcomes are prevented with GP treatment, thus proving our hypothesis that GP has protective effects at least on age-associated behavioral deficits but not on cognitive deficits. Lack of a more rigorous mechanistic analysis is a limitation of this study. Nevertheless, we believe that our study has provided the groundwork needed to more actively pursue CTGC powder as a potential nutraceutical. We suggest that CTGC-prepared GP with its antioxidant properties has a good potential to be considered as an antianxiety agent. This proposition if proved accurate would have immense therapeutic benefit for our aging population and also to promote healthy aging. Other laboratories must test this powder so that present results can be independently verified before its true potential is revealed.

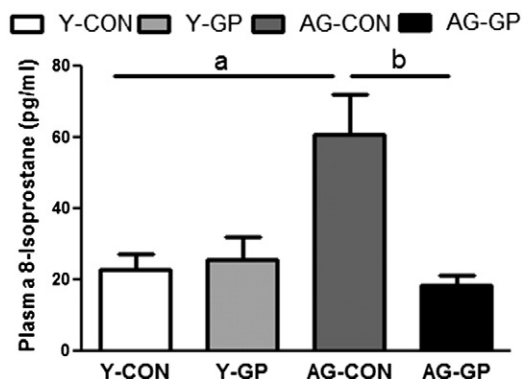


Fig. 6 – Examination of 8-isoprostane levels in plasma. The level of plasma 8-isoprostane was measured using an EIA kit (Cayman). A and B Means without a common letter differ ($P < .05$). Bars represent means \pm SEMs; $n = 8$ –10 rats/group.

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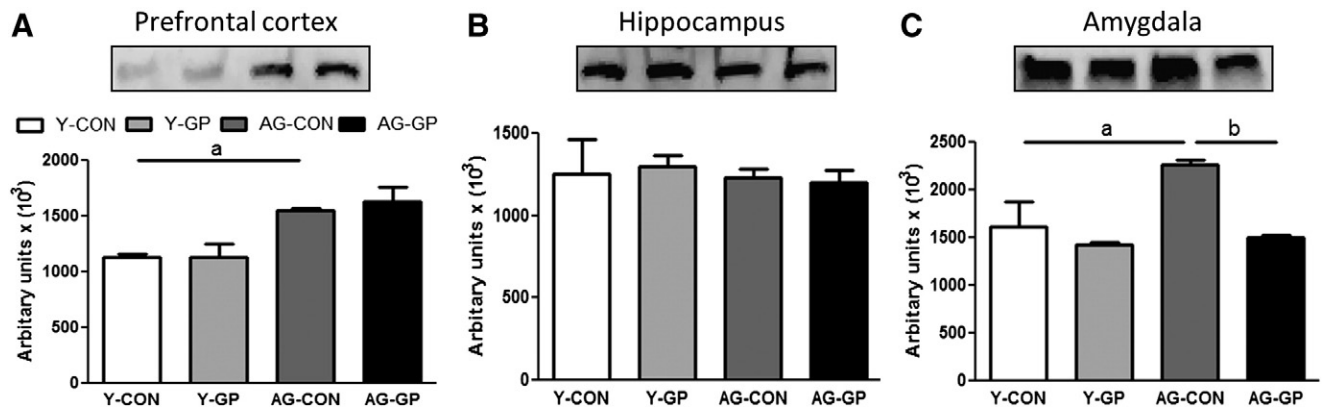


Fig. 7 – Analysis of markers of oxidative stress protein carbonylation. The level of protein carbonylation in the prefrontal cortex (A), hippocampus (B), and amygdala (C) was measured with an OxyBlot kit (Millipore). A and B, Means without a common letter differ ($P < .05$). Bars represent means \pm SEMs; $n = 8$ –10 rats/group.

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