

# Effect of Chlorogenic Acid on Sperm Parameters, DNA Integrity and Malondialdehyde of Carbamazepine - Consuming Epileptic Mice

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## Abstract

**Background:** Recently, administration of antiepilepsy drugs (AED) has become very common. This study aimed to assess the impacts of chlorogenic acid(CA) on parameters, features of chromatin and DNA integrity of spermatozoa and assess the Malondialdehyde (MDA) in carbamazepine (CBZ)-consuming epileptic mice.

**Materials and Methods:** thirty-six adult male NMRI mice were randomly selected into six groups. Group I (Control), received basal diet; group II, pentylenetetrazol (PTZ); group III, PTZ+carbamazepine; group IV, CA; group V, PTZ+CA; group VI, PTZ+CBZ+CA. Finally, removal of caudal epididymis was done and placed in 1mL Ham'sF10 medium at 37C for 15 min. Then, analysis of sperm parameters was executed. Toluidine blue (TB), chromomycinA3 (CMA3), aniline blue (AB) and TUNLE were applied for assessment of sperm chromatin quality. Also, lipid peroxidation and total antioxidant capability were measured by MDA test.

**Results:** A significant increase was evident in sperm parameters in the CA, PTZ+CA and PTZ+CBZ+CA groups in comparison with the control and PTZ groups. About AB, TB, CMA3 and TUNEL tests, there was a significant increase in the PTZ group in comparison with the PTZ group. MDA test showed significant decreasing in the PTZ group compared to all groups. Also, the PTZ+CBZ+CA group had a significant increase compared to the control, PTZ, PTZ+CBZ and PTZ+CA groups

**Conclusion:** Although epilepsy can have deleterious effects on sperm fertility and DNA integrity, the CBZ and CA administration has positive result on sperm parameters, sperm chromatin abnormalities, and MDA in mice with epilepsy.

**Keywords:** Chlorogenic Acid, Sperm Parameters, Chromatin Quality, Malondialdehyde, Carbamazepine, Epileptic Mice.

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## INTRODUCTION

Epilepsy is one of the most widespread diseases of the neural system. Approximately 50 million people worldwide are affected by this disease (1). In Belgium, About 100000 people have epilepsy (2). The impact of antiepileptic drugs in the progression of endocrine disorders in people with epilepsy is controversial. Infertility disorders are a prevalent complication of epilepsy in men (3, 4). Epilepsy can interfere with the regulation of the secretion of reproductive

hormones (5). According to studies of active epilepsy during reproduction, it may lead to reproductive disorders and the cause of infertility (6). Sexual dysfunction in patients with epilepsy has several causes. This is the pathophysiological result of epilepsy and associated anticonvulsant therapies (7). In general, it is impossible to individualize between sexual disorders caused by epilepsy and usable disorders affected by medication. Expectant epileptic discharge disrupts nerve and glandular functions and alters the secretion of hypothalamic and pituitary hormones by

increasing prolactin and fluctuating LH. Some antiepileptic drugs (AEDs) may reduce endocrine disorders, which can alter male reproductive functions (7).

Epilepsy and AEDs might contributed in bring about these complications, nevertheless, the exact mechanisms are not distinctly identified and the straight effects of epilepsy contrary to AED are difficult to distinguish (8).

Carbamazepine (CBZ) is one of the main drugs used to treat epilepsy and neuropathic pain (9). Its function includes maintenance of the inactive phase of voltage-gated sodium channels, amplification of gamma aminobutyric acid (GABA) receptors as GABA agonists, and the effect of serotonin release (10, 11). First CBZ is metabolized by CYP450 3A4 in the liver. CBZ and its metabolite are recognized to induce oxidative stress and reactive oxygen species (ROS) (12). The reproductive system is more affected by CBZ toxicity than other body systems. CBZ administration reduces sperm concentration, testicular heaviness, testosterone, FSH and LH hormones (11-8). In men taking CBZ, there is a gradual increase in the level of globulin-bound sex hormones (SHBG) and a decrease in the ratio of FAI to a decrease in bioactive androgens, and it has been indicated that these changes cause a decrease in sexual activity (13).

Chlorogenic acid (CA) is broadly found in coffee, tea, herbs and many fruits and as an important polyphenolic compound in the diet with a range of biological activities such as antioxidant and anti-inflammatory (14). CA also informed as 5-O-caffeoylquinic acid (5-CQA) (IUPAC numbering) or 3-CQA (pre-IUPAC numbering) is an ester formed from cinnamic acids and quinic acid (15). CA meliorates some parameters of sperm as well as motility, viability and plasma membrane integrity. According to our research, there have been finite studies on the effect of CA as an antioxidant on male infertility and sperm quality and function (16).

Due to the high incidence of epilepsy and the importance of fertility, the objective of our survey was to investigate the preserve impacts of CA on sperm parameters, chromatin quality, DNA integrity and oxidative stress in carbamazepine-treated epileptic mice.

## MATERIALS AND METHODS

Thirty-six adult male NMRI mice were accidentally selected into six groups: Group I (Control), received basal diet; group II, received basal diet+pentylentetrazol (PTZ) (35mg/kg, intraperitoneal every 48 hours); group III, received received basal diet+PTZ+CBZ (30mg/kg, by intra-gastric gavage every day); group IV, received received basal diet+CA (50 mg/kg, by intra-gastric gavage every day); group V, received basal diet+PTZ+CBZ; group VI, received basal diet+PTZ+CBZ+CA.

Finally, removal of caudal epididymis was done and placed in 1mL Ham'sF10 medium at 37C for 15 min. Then, examination of sperm parameters (count, motility, viability

and morphology) was executed according to the World Health Organization (WHO) guidelines (Organization WHO 2010). Toluidine blue (TB), chromomycinA3 (CMA3) and aniline blue (AB) for assessment of sperm chromatin quality and DNA integrity, TUNLE were applied for apoptosis. Also, lipid peroxidation and total antioxidant capability were measured by malondialdehyde (MDA) analysis.

### Sperm count

The average number of normal sperm in 10 fields of view of a light model microscope (Olympus / 3H - Z) made in Japan was examined and a Neubauer hemocytometer was used to count the number of sperms. For this purpose, a drop of diluted sample was placed on the slide and then the squares of the white blood cells (16 cells) were counted accurately and the number of sperms calculated was multiplied by 10<sup>6</sup> to get the total sperm count(17).

### Sperm motility

To evaluate sperm motility, 10 µl of each sample was taken by sampler and emptied into the Mackler chamber. In microscopic examination of specimens with 20% magnification according to WHO criteria, sperms were divided into 3 groups: progressive motility, in situ motility (non-progressive) and non-motile(18), Using a light microscope, 200 sperm were examined and expressed as a percentage(19).

### Sperm viability

Eosin-nigrosin (EN) staining was applied to assess survival. Spotted sperm are considered dead pink / red heads. At least 200 sperm were counted in each patient with a magnification of 1000(19).

### Sperm morphology

To study the morphology of sperm, after placing a drop of culture medium containing sperm on the slide, a smear was prepared with another slide, and then the smear was fixed in a mixture of ether and alcohol 96% (1: 1). Next, the slides were stained with Papanicolaou staining. In this staining, the sperm nucleus was blue, the acrosome and tail of the sperm were pink, and the dorsal part of the sperm was dark blue. For each sample, 100 sperm were examined by 100% magnification of a light microscope. Sperms were expressed as a percentage based on morphology(20).

### Aniline blue staining

Aniline blue staining was applied to evaluate the chromatin density of sperm samples. In this method, prepared and dried smears were stabilized at air temperature using 4% formalin. They were then washed with water and stained in 5% AB in 4% acetic acid solution (pH 3.5). Each fixation and staining steps were carried out at room temperature for

5 minutes. The slides were washed with water, dried at room temperature and evaluated using a light microscope. At least 200 sperm were counted and examined. Dark-stained sperm were considered immature sperm with excess histone and abnormality in chromatin(21).

**Toluidine blue staining**

The status of chromatin were assessed by applying a thin smear was prepared on the slide. The smears were dried at air temperature and fixed with 96% ethanol-acetone (1: 1) at 4°C for one hour. The slides were then placed in 0.1 N HCl at 4 ° C for 5 minutes, followed by washing 3 times with distilled water for 2 minutes. Then toluidine blue staining 005 % were done and the slides were kept in the room temperature for 5 minutes. Toluidine blue dye was used to assess the chromatin. The head of the sperm is light blue with healthy chromatin and those with fragmented or abnormal chromatin are dark purple. A total of 200 sperm per slide was observed and evaluated using a light microscope(21).

**Chromomycin A3 (CMA3) staining**

The semen sample is fixed in Carnoy solution for 10 minutes at 48°C and a smear is prepared from it. Each slide was stained with 100 µl of CMA3 solution (Sigma) (0.25 mg / ml McIlvain Buffer containing 10 mM magnesium chloride at pH=7) for 20 minutes to remove residue. Excess dye washes the slides in PBS solution and examines them with a 100 magnification fluorescence microscope. About 200 sperm are tested for each sample. Heads of sperm that are deficient in protamine are bright yellow (CMA3<sup>+</sup>), which is reported as the percentage of CMA3<sup>+</sup> sperms, and those that are naturally occurring are light yellow (CMA3<sup>-</sup>), and as the percentage of CMA3<sup>-</sup> sperms is reporting(20, 21).

**TUNEL assay**

First, the semen was washed with PBS solution and stained by preparing a smear on the slide according to the instructions of the TUNEL kit. According to this method, the fluorescent dye is attached to the ends of broken DNA fragments by the rTdT enzyme, and fluorescein-12-dUTP marks the DNA. Examination of the fluorescent green by a 100-magnitude fluorescence microscope (BX51; Olympus; Japan) in the posterior region of the sperm head indicates TUNEL<sup>+</sup> (DNA-damaged sperm) and red indicates TUNEL<sup>-</sup> sperm (DNA sperm is healthy) which is reported as a percentage. About 200 sperm are tested for each sample(22).

**Malondialdehyde (MDA) assay**

100 µl of the test specimens were transferred to the Eppendorf tubes and about 400 µl of the trichloroacetic acid solution was added. About 10 microliters of hydroxytoluene was added to the solution and then centrifuged at 3000 g for 10 minutes. Then 500 µl of the supernatant solution was

removed and about 400 µl of thiobarbituric acid solution was added to the solution and kept at 95 ° C in a boiling water bath for one hour. The samples were then refrigerated for 15 minutes to cool. The samples were then centrifuged again for 10 minutes at 4000 g and the optical absorption of the supernatant at 532 nm was examined by spectrophotometer. Finally, the concentration of MDA was calculated according to the standard diagram (23).

**STATISTICAL ANALYSIS**

Obtained data were demonstrated as mean+standard deviation (SD). To assess the significant difference between the 6 groups One-way ANOVA test was used and Tukey post-test was used to define diversity between the two groups. P<0.05 was considered significant.

**RESULTS**

**Sperm parameters analysis**

Table 1 shows the statistical analysis of the various sperm parameters between the 6 groups. This table reveals that sperm count, sperm motility (Progressive, Non progressive and Immotile), sperm viability and morphology were significantly different (P<0.05) between Groups.

Table 1: Sperm parameters in different groups

Variables	control	PTZ	PTZ+CBZ	CA	PTZ+CA	PTZ+CBZ+CA
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
<b>Count</b> (×10 <sup>6</sup> )	35.66±2.87bcd	22.32±5.21aef	23.84±2.45ae	140±11.22ade	100.6±1.37abcd	30.56±3.52abe
<b>Progressive motility</b>	56.33±5.13bc	28.71±6.67adef	32.62±6.71adf	54±4.08bce	37.5±5.34abdef	55.5±1.063bce
<b>Non-progressive motility</b>	26±6.55ce	26±4.89	29.75±5.8a	27±5.47	30.38±9.45a	20.75±4.27
<b>Immotile</b>	25.5±5.8bc	45.43±6.18acdef	38.12±6.12abf	22.75±5.56bcde	31.5±8.41bdf	17.67±1.52bce
<b>Viability</b>	68.33±17.09bce	25.43±1.01acdef	46.12±13.54abd	81.75±5.43bcde	47.5±16.02abd	54±14.72bd
<b>Morphology</b>	60±3.46bc	39.14±6.28acdef	55.12±2.93ab	71.75±11.47b	59.88±7.12b	59.25±5.2b

- a: Significant in comparison with control group
- b: Significant in comparison with PTZ group
- c: Significant in comparison with PTZ+CBZ group
- d: Significant in comparison with CA group
- e: Significant in comparison with PTZ+CA group
- f: Significant in comparison with PTX+CBZ+CA group

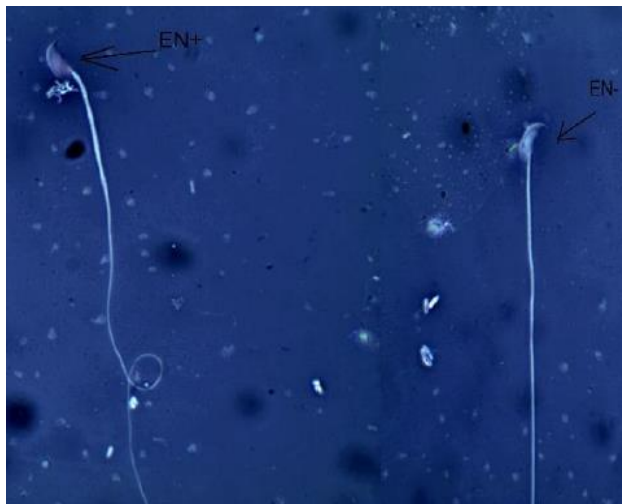


Figure 1: the EN+ indicates a dead sperm (colored sperm) and the EN- indicates live sperm (non-colored sperm). Eosin-nigrosine (EN) staining, ×100 eyepiece magnification.



Figure 2: The PAPA+ indicates a normal spermatozoon and the PAPA- indicates abnormal spermatozoon. Papanicolaou staining, ×100 eyepiece magnification.

### Sperm chromatin/ DNA evaluation

Table 2 demonstrated the outcome of sperm chromatin and DNA integrity analysis between 6 Groups. Table 2 showed the result of examination of sperm chromatin and DNA integrity between 6 Groups. In AB staining, the percentages of normal sperm (unstained or pale blue) and abnormal sperm (dark blue) were stated. In TB staining, the quality of sperm chromatin was assessed according to metachromatic staining of sperm heads in following scores: 0, good chromatin (light blue); 1, mild abnormal chromatin (dark blue); 3-4, severe chromatin abnormality (violet and purple). So, the sum of sperm with score 1, 2 and 3 was considered as sperm with abnormal chromatin, whereas score 0 sperm were considered as sperm with normal chromatin. In CMA3 staining, the percentages of abnormal sperm (bright yellow) and normal (yellowish green) were stated.

Table 2: Evaluation of sperm chromatin/DNA in different groups.

Variables	control	PTZ	PTZ+CBZ	CA	PTZ+CA	PTZ+CBZ+CA
	Mean ±SD	Mean±SD	Mean±SD	Mean ±SD	Mean±SD	Mean±SD
AB	62.25 ±13.74b	24.86±8.8acd ef	48.75±16.8bf	65±3.55b	58.88±15.04bf	77.67±7.09bce
TB	63.25 ±6.7bce	21.57±8.01ac de	37.88±6.15ab de	75.5±7.32bc	55.38±12.09abc	80±9bc de
CM A3	67±13.9b	39.29±9.17adf	56.12±16.79d	81.75±8.05bce	53.12±18.7d	75.67±5.85b

- a: Significant in comparison with control group
- b: Significant in comparison with PTZ group
- c: Significant in comparison with PTZ+CBZ group
- d: Significant in comparison with CA group
- e: Significant in comparison with PTZ+CA group
- f: Significant in comparison with PTX+CBZ+CA group

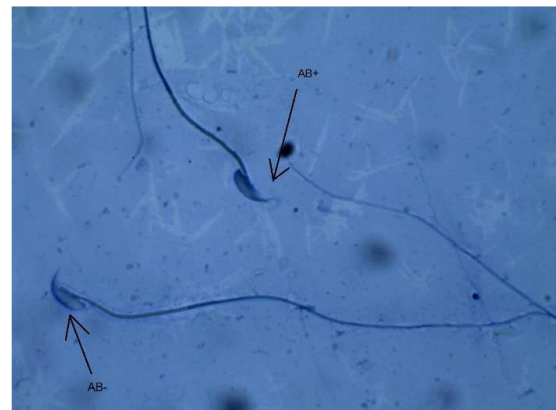


Figure 3: Abnormal sperm (AB+) and normal sperm (AB-). AB staining, ×100 eyepiece magnification.

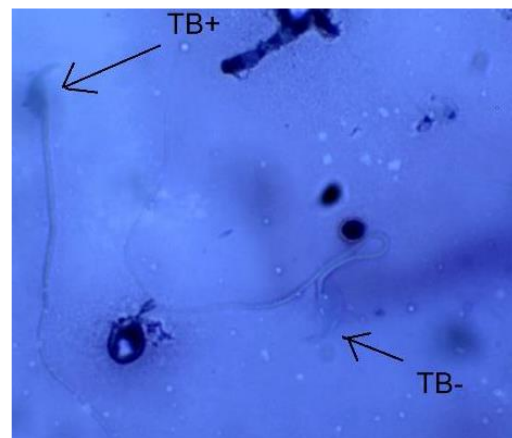


Figure 4: Sperm with abnormal chromatin (TB+) and sperm with normal chromatin (TB-). TB staining, ×100 eyepiece magnification

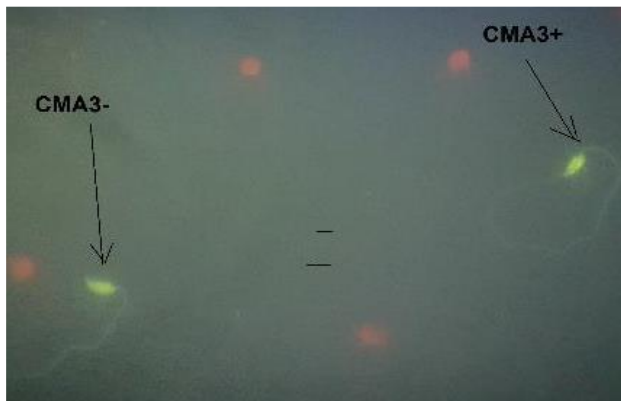


Figure 5: Sperm with protamine deficiency (CMA3+) and sperm with normal protamine content (CMA3-). CMA3 staining, ×100 eyepiece magnification.

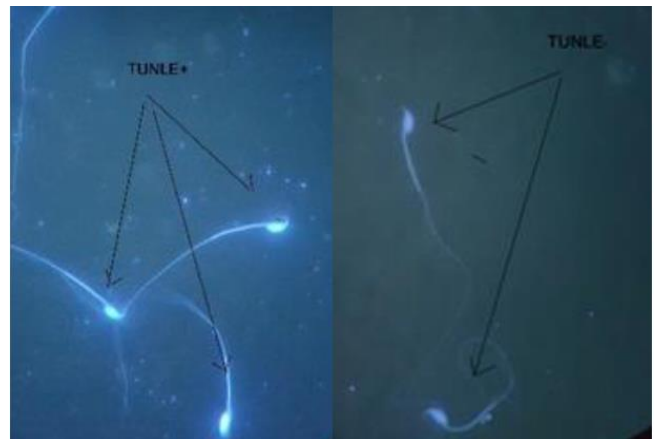


Figure 6: TUNEL+ indicates normal DNA (non-apoptotic sperm) and TUNEL- indicates damaged DNA (apoptotic sperm). TUNEL assay, ×100 eyepiece magnification

### Apoptosis and oxidative stress evaluation

Table 3 presents the outcome of examination of sperm apoptosis and MDA as a marker for oxidative stress between Groups. It should be noted that in TUNEL staining, A light-colored sperm nucleus identifies sperm with fragmented DNA, and a pale sperm nucleus identifies sperm with healthy DNA.

Table 3: The TUNEL and MDA evaluation in different groups.

Variable	control	PTZ	PTZ+CBZ	CA	PTZ+CA	PTZ+CBZ+CA
	Mean ±SD	Mean ±SD	Mean±SD	Mean ±SD	Mean ±SD	Mean±SD
TUNEL	96.25±2.75bcde	88±2.82acdef	91.75±2.86abcd	99.75±1.89bcde	92.38±1.92bd	95±1b
MDA	25±2.94bcdef	52±5.97acdef	35±5.39abdf	10±2.16abcde	38±4.24abdf	16±2abce

- a: Significant control group
- b: Significant in comparison with PTZ group
- c: Significant in comparison with PTZ+CBZ group
- d: Significant in comparison with CA group
- e: Significant in comparison with PTZ+CA group
- f: Significant in comparison with PTX+CBZ+CA group

### DISCUSSION

There are complex connections between epilepsy, AEDs, and hormones. Endocrine abnormalities are more common in people who have epilepsy problem. Abnormalities are often described for the sex steroid hormone axis, which is seen ordinary as sexual dysfunction in women and men with epilepsy and low fertility (24). According to the research of Mehrab Nasab et al., Who found that epilepsy reduces sperm parameters such as motility by reducing testosterone. In the present study, PTZ-induced epilepsy also decreased sperm parameters and chromatin quality and increased apoptosis. It has also been observed in the group of epileptic mice that the amount of oxidative stress has increased, which can be concluded that the increase in oxidative stress has reduced the sperm quality in epileptic mice (25). Low levels of dehydroepiandrosterone sulfate (DHEAS), motility and morphology of sperm have been reported in men with CBZ epilepsy (26). By reducing oxidative stress, CA improves sperm parameters and quality of chromatin and reduces apoptosis in PTZ+CA and PTZ+CBZ+CA groups. It has recently been discovered that CA has purposive efficacy on cell function and animal health, as well as improving the quality of animal sperm(27, 28). So far, few research has been done on the effect of CA on sperm, especially sperm chromatin quality. Our results showed that CA as an antioxidant reduced oxidative stress and improved sperm parameters, DNA integrity and apoptosis in epileptic mice.

Significant amounts of literature have been published that support the theory of a link between increased DNA damage and infertility, and in 2015 Shina concluded that DNA fragmentation testing should be part of the normal diagnosis of male infertility (29). With the expansion of research in the field, various studies show that the origin of DNA damage is very different. There is a relationship between increased DNA damage and inadvertent effects during spermatogenesis, increased ROS, sperm collection methods,

storage temperature, age, testicular temperature, and drug response (30). Therefore, DNA damage can occur in several stages. This may have contributed to the blurring of the DNA division image. One theory is that DNA undergoes destructive events during spermatogenesis. This could include nicks in the backbone of the DNA or poor packaging of the chromatin during the exchange of histones. eventually, the weakened DNA is more sensitive to external stressors such as temperature, drug and ROS(31). In the present study, it is possible that the increase in ROS in the PTZ-induced epileptic group caused a defect in sperm DNA, and on the other hand, CA in the PTZ+CA and PTZ+CBZ+CA groups increased chromatin quality by reducing the production of ROS. CA by modulating the accumulation of reactive ROS and regulating the regulation of the main enzymes and proteins involved in cell apoptosis can mediate oxidative stress and reduce cellular apoptosis caused by various stressors and ROS (32). In this study, CA also reduced sperm apoptosis in all groups consuming CA.

AEDs have been shown to cause a variety of hormonal disorders. In particular, the use of liver enzyme-inducing AEDs, such as phenytoin and CBZ, which increase SHBG concentrations, This increase leads to a decrease in testosterone activity, which can lead to a decrease in strength and thus a decrease in fertility (33). According to the some of examinations, men with epilepsy treated with CBZ have altered semen quality compared to healthy individuals (34, 35). Asadi-Pooya et al Examining the efficacy of CBZ on sperm quality in men who suffering from epilepsy showed that CBZ caused changes in sperm quality including abnormalities in sperm concentration, morphology and motility (8). Most animal examinations have shown the effect of AEDs such as CBZ on sperm and infertility without induction of epilepsy, which in these studies has shown that these drugs have somewhat reduced sperm quality. However, in the present study, epilepsy induction showed that the parameters and quality of sperm chromatin were lower in the group that used PTZ than in the group that used CBZ with PTZ. Therefore, it can be concluded that CBZ indirectly improves the parameters and quality of chromatin by improving epilepsy, as well as reducing the rate of apoptosis and oxidative stress in sperm.

## CONCLUSION

According to our result epilepsy reduces sperm parameters, chromatin quality and increases sperm apoptosis and oxidative stress. CBZ increased sperm quality by improving epilepsy. CA as an antioxidant also improved sperm parameters, sperm chromatin and apoptosis in epileptic mice by reducing oxidative stress.

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