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Research Article

DNA fragmentation in leukocytes following subacute low-dose nerve agent exposure

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Abstract. The objective of the present study was to determine levels of DNA fragmentation in blood leukocytes from guinea pigs by single-cell gel electrophoresis (comet assay) after exposure to the chemical warfare nerve agent (CWNA), soman, at doses ranging from 0.1 LD_{50} to 0.4 LD_{50}, once per day for either 5 or 10 days. Post-exposure recovery periods ranged from 0 to 17 days. Leukocytes were imaged from each animal, and the images analyzed by computer. Data obtained for exposure to soman demonstrated significant increases in DNA fragmentation in circulating leukocytes in CWNA-treated guinea pigs compared with saline-injected control animals at all doses and time points examined. Notably, significantly increased DNA fragmentation was observed in leukocytes 17 days after cessation of soman exposure. Our findings demonstrate that leukocyte DNA fragmentation assays may provide a sensitive biomarker for low-dose CWNA exposure.

Key words. Biomarker; soman; blood; guinea pig; leukocyte; immune system; comet assay analysis.

Introduction

Much controversy has surrounded the possible involvement of chemical warfare nerve agents (CWNAs) in the etiology of 'Gulf War Syndrome' among armed forces personnel deployed to the Persian Gulf in 1990 [1]. Despite the fact that US military surveillance teams detected no exposures to chemical weapons, other countries claim to have detected low-level gaseous nerve agents [2]. More recently, the potential for terrorists to use nerve agents became a reality when sarin gas was released in a Japanese subway in March 1995. Such chemical terrorist attacks pose a definite threat to both civilians and military personnel in the US and overseas and, as such, research into methods of protection against nerve agent-induced tissue and brain injury has become of paramount importance.

Classical nerve agents, such as soman, sarin, tabun and VX, are extremely toxic, irreversible cholinesterase inhibitors that can be used in military operations, or by terrorists, to kill, incapacitate or seriously injure opponents. Organophosphates exert their effects by inactivating the enzyme acetylcholinesterase, causing accumulation of acetylcholine at neuronal synapses and neuromuscular junctions, to result in hyperactivity of the cholinergic system, and tetany of skeletal muscles, including the di-

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Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulation relating to animals and experiments involving animals, and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, NIH publication 85–23. The views of the authors do not purport to reflect the position of the Department of the Army or the Department of Defense, (para 4-3), AR 360-5.

aphragm [3]. Traditionally, anticholinergic compounds have been demonstrated to provide variable degrees of neuroprotection against organophosphates [4]. Recent studies have also attributed involvement of NMDA receptor modulation, in conjunction with the anticholinergic properties of neuroprotective drugs like caramiphen, against organophosphate toxicity [5].

Neuronal DNA fragmentation in response to central nervous system (CNS) injury is a well-studied phenomenon, but leukocyte DNA fragmentation in response to injury is less well studied. Similarly, although cellular DNA fragmentation or apoptosis is a well-documented process following traumatic or ischemic injury, its involvement in nerve agent-induced cellular degeneration remains to be elucidated. In particular, the effects low-level nerve agent exposure might have on leukocyte function and integrity are not known. In this report, we quantify the level of DNA fragmentation in circulating leukocytes associated with low-dose soman-mediated injury using the single-cell electrophoresis 'comet' assay to determine if such assays could be used as a biomarker for low-dose CWNA exposure.

Materials and methods

Male guinea pigs (400–500 gm, n = 3 to 4 per group, total = 47) were injected with saline, or 0.1 LD_{50}, 0.2 LD_{50} or 0.4 LD_{50} soman (1 ml/kg) dissolved in sterile physiological saline, Monday through Friday, once a day at 0800 h for either 5 or 10 days (animals were not injected on the weekend). At 1200 h on day 5, day 12 or day 29, animals were anesthetized with pentobarbital (325 mg/kg) and sacrificed by decapitation. Blood was collected in tubes containing EDTA, and kept on ice until processed for comet analysis.

Whole blood (100 µl) from each animal was added to 1 ml of ice-cold Ca^{2+}, Mg^{2+} free PBS with 20 mM EDTA. The mixtures were centrifuged at 1000 g for 10 min to remove plasma, and the cell pellet was resuspended in 1 ml of the same solution. Cells were washed by this method two more times to remove cell debris. The final pellet was suspended in 1 ml of ice-cold PBS with 20 mM EDTA, and 50 µl was removed and combined with 500 µl of low melting-point agarose (LMAgarose; Trevigen, Gaithersburg, Md.) warmed to 42 °C. For the rest of the procedure, the manufacturer's (Trevigen) protocol was used for the comet assay. Briefly, a 50-µl aliquot of this mixture was transferred to specially treated slides and allowed to cool. Slides were then immersed in ice-cold lysis solution (pH 9.6; Trevigen) and incubated for 30 min at 4 °C. Slides were then moved to an alkali buffer solution (0.6 g NaOH and 100 µl 0.5 M EDTA in 50 ml of purified water, pH 12.8) for 20–30 min at room temperature. Slides were then washed twice in Tris-borate EDTA solution (TBE buffer, pH 8.4), 5 min each, and then placed in the electrophoresis chamber. Electrophoresis was run for 10 min at 25 mV in TBE buffer. Slides were removed from the apparatus and were fixed for 5 min with ice-cold 100% MeOH, followed by 5 min in ethanol, and then dried in the dark at room temperature.

For quantification of DNA fragmentation, 30 leukocytes were imaged per animal, with three to four animals in each group (90–120 individual leukocytes per group). Specially designed comet analysis software (Loats Associates, Westminster, Md.) was used to analyze the degree of DNA fragmentation in individual leukocytes. Slides were covered with 50 µl of 0.01% SYBR Green (Trevigen) in Tris-EDTA buffer and imaged with a monochrome digital camera connected to an Olympus BX60 fluorescent microscope using a 20 × objective.

The comet assay does not provide a measure of the number of cells that are apoptotic out of the population of imaged leukocytes. Therefore, a direct count of apoptotic cells was undertaken. For each animal, 500 cells were counted, and the percentage that was clearly apoptotic was tabulated. Leukocytes were not scored as apoptotic unless there was a clear comet head and comet tail, as well as granularity of the fluorescent signal in the nucleus.

Results

Figure 1 presents the data obtained from counting over 22,000 cells from the 47 animals in the current study. Leukocytes with DNA fragmentation were rare in blood samples from the saline-injected animals, but were significantly more numerous in blood from soman-injected animals. The representative images in figure 2 show a normal leukocyte from a saline-injected animal (fig. 2A), as compared with a leukocyte taken from a soman-injected animal (fig. 2B) after 5 days of exposure. Typically, normal leukocytes had smooth edges, and little or no DNA was observed in the comet tail. When DNA fragmentation was present, the fluorescent signal in the nucleus appeared granular, the edges of the nucleus were uneven, and some of the DNA had migrated out of the nucleus into the comet tail (a hallmark of apoptosis). As seen in figure 1, a dose response was observed for all three groups of animals in the study, where increasing soman exposure from 0.1 LD_{50} to 0.4 LD_{50} resulted in an increase in DNA fragmentation. The maximal effect was observed in the 0.4 LD_{50} group with 10 days of soman exposure, followed by 17 days of recovery, where over 17% of leukocytes had DNA fragmentation, compared with just over 6% for the saline control group.

DNA fragmentation in circulating leukocytes was significantly increased in blood samples from all soman-treated animals, at all time points, as shown in figures 3–5. Fig-

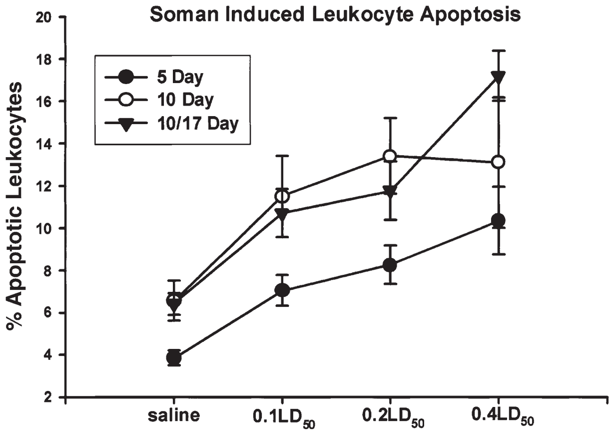

Figure 1. Percentage of leukocytes exhibiting apoptosis after soman exposure at four doses and three time points. Three to four guinea pigs were in each group, and 250–500 leukocytes were counted per animal. Only cells which exhibited a clear comet head and tail, as well as nuclear granularity, were counted as apoptotic. Error bars represent the SE of the mean (n = 3 to 4 per group, total = 47 animals).

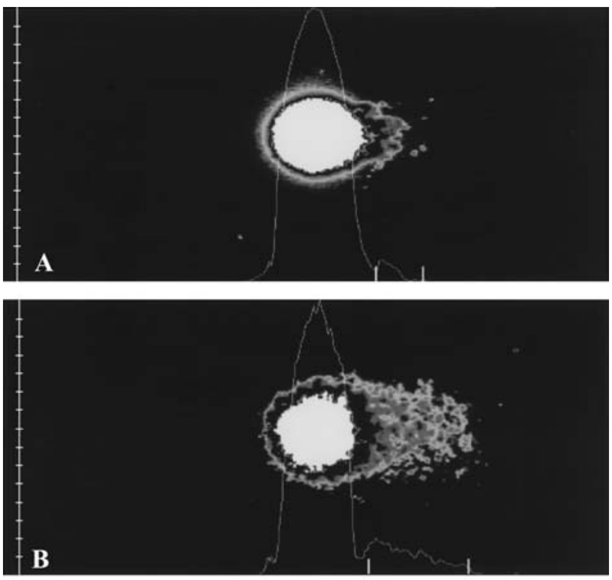

Figure 2. Representative images of leukocytes from control (*A*) and soman-injected (*B*) guinea pigs. The fluorescence intensity for each vertical strip of pixels in the image is summed and shown as an overlaid graph. Tail length is a measure of the linear length of the comet tails, while % DNA is a measure of the area under the intensity signal for the cell nucleus compared with the area under the intensity signal for the comet tail. The moment arm is a measure of the average DNA migration from the nucleus.

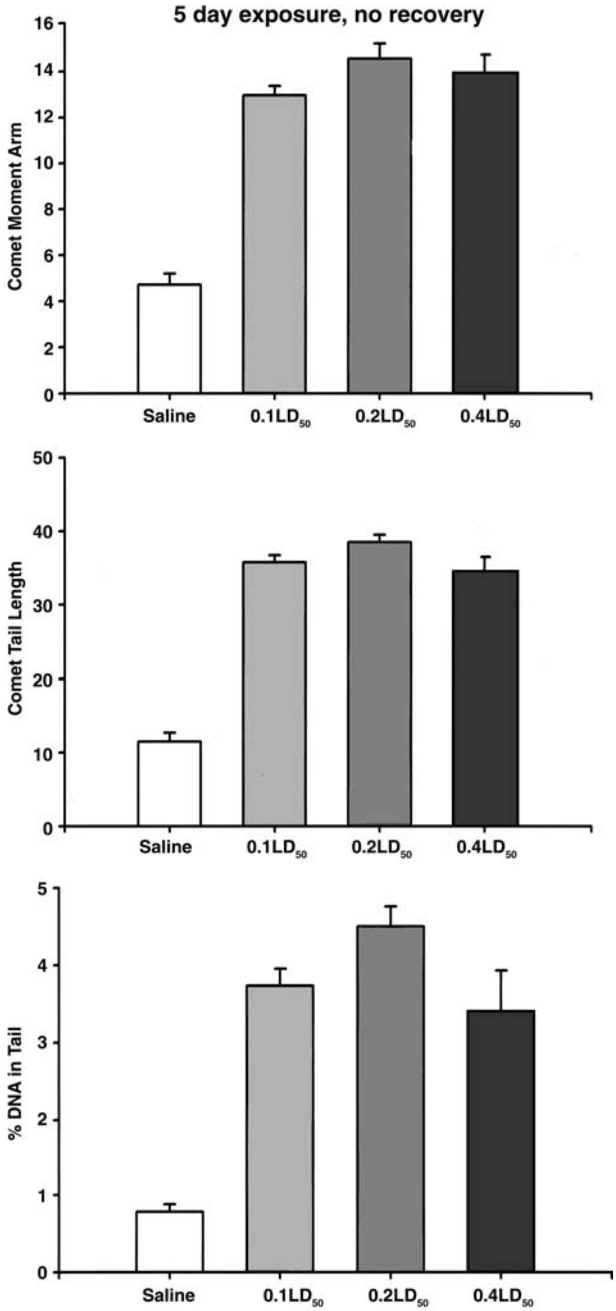

Figure 3. Average measures of apoptosis and DNA damage taken after 5 days of soman exposure, with no recovery period. All experimental values were significantly different from the control group (t test, $p < 0.001$). Error bars represent the SE of the mean.

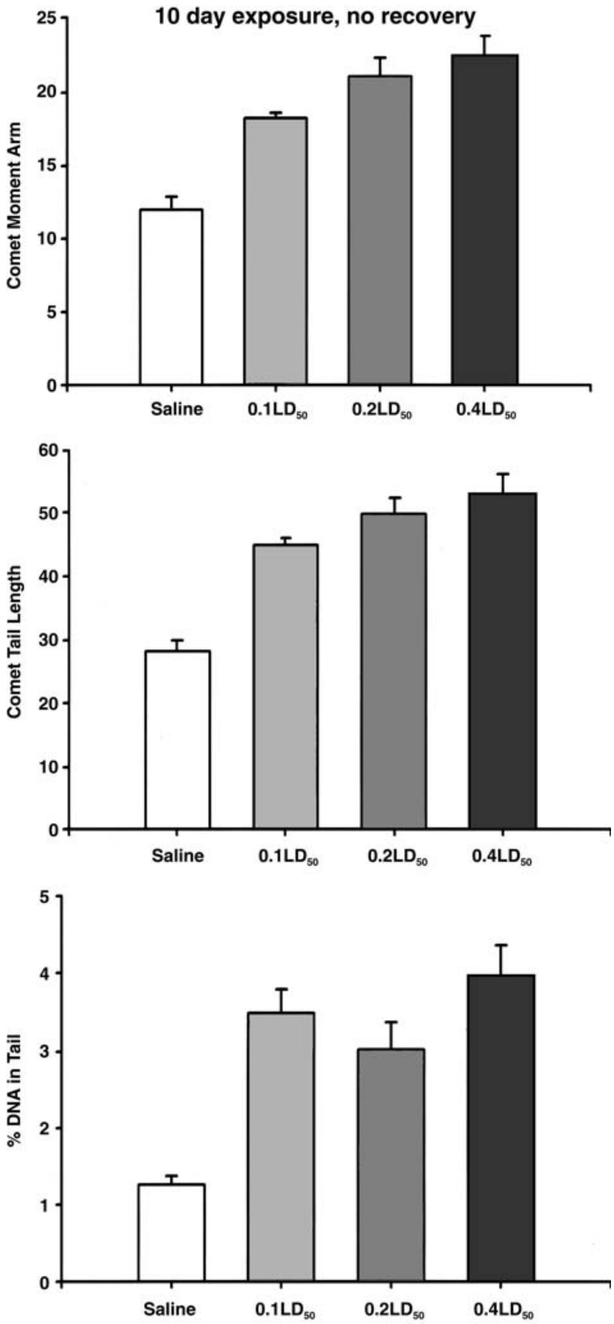

Figure 4. Measures of DNA damage after 10 days of soman exposure with no recovery period. All experimental values were significantly different from controls ($p < 0.01$). Error bars represent the SE of the mean.

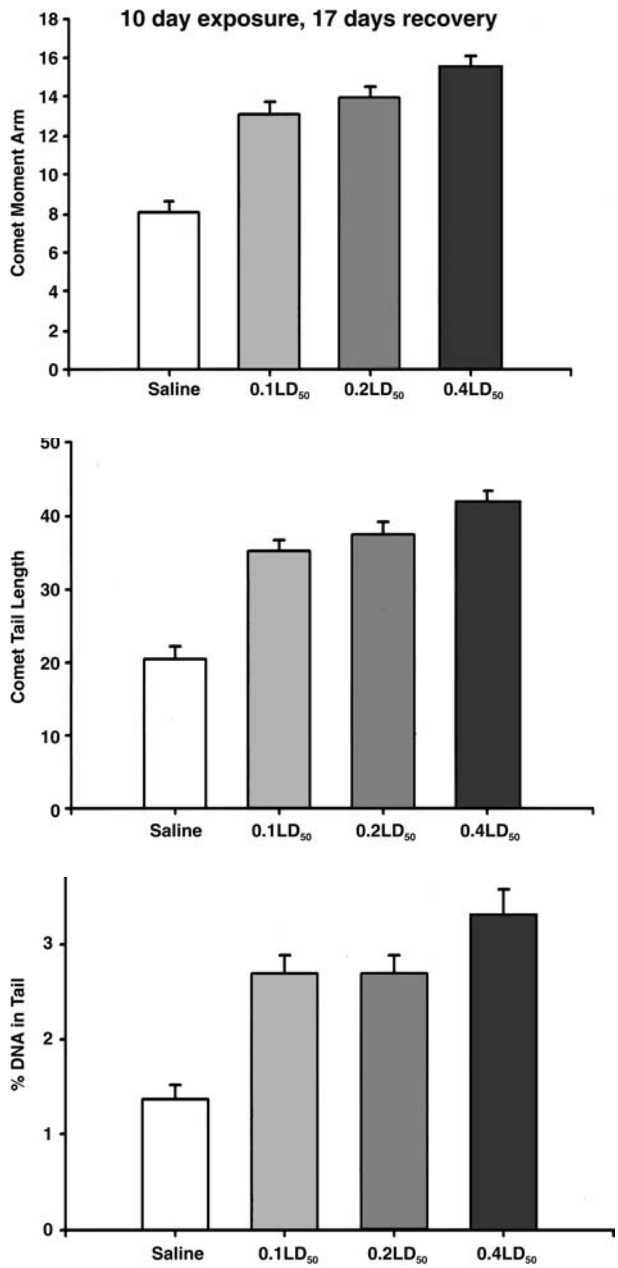

Figure 5. Comet analysis after 10 days of soman exposure, with the blood being collected 17 days after the last exposure. All measures were significantly different from controls ($p < 0.001$). Error bars represent the SE of the mean.

ure 3 shows the increase in several measures of DNA fragmentation after 5 days exposure to three doses of soman, compared with saline-injected controls. The average moment arm, a measure of the level of DNA migration into the comet tail, increased from 4.68 ± 0.53 in control animals to 12.91 ± 0.38 in the 0.1 LD_{50} group, 14.54 ± 0.58 in the 0.2 LD_{50} group and 13.8 ± 0.81 in the 0.4 LD_{50} group. The average percentage of DNA in the comet tails in the animals exposed to soman for 5 days increased from 0.79 ± 0.11 in saline controls to 3.72 ± 0.25 in the 0.1 LD_{50} group to 4.68 ± 0.62 in the 0.2 LD_{50} group and 3.40 ± 0.52 in the 0.4 LD_{50} injected group. The average comet tail length, a measure of DNA fragmentation, was increased from 11.35 ± 1.34 in control animals to 35.58 ± 1.13 in the 0.1 LD_{50} group, 39.41 ± 1.53 in the 0.2 LD_{50} group, and 34.33 ± 2.10 in the 0.4 LD_{50} group.

Figure 4 shows several measures of DNA fragmentation in leukocytes collected from guinea pigs injected with soman for 10 days (5 injection days, 2 days recovery, followed by 5 more days of injections). The average comet tail moment arm increased from 11.95 ± 0.83 for the control group to 18.18 ± 0.43 in the 0.1 LD_{50} group, 21.04 ± 1.26 in the 0.2 LD_{50} group, and 22.38 ± 1.39 for the 0.4 LD_{50} group. The average percentage DNA in the comet tails increased from 1.26 ± 0.10 in the control animals to 3.48 ± 0.35 in the 0.1LD_{50} group, 3.00 ± 0.37 in the 0.2 LD_{50} group, and 3.96 ± 0.41 in the 0.4 LD_{50} group. The average comet tail length increased from 28.02 ± 1.90 in the control group to 45.03 ± 1.12 in the 0.1 LD_{50} group, 49.76 ± 2.74 in the 0.2 LD_{50} group, and 53.08 ± 3.16 in the 0.4 LD_{50} group.

Figure 5 shows the average comet measures for animals tested on day 29 (5 days exposure, 2 days recovery, 5 more days of exposure, followed by 17 days recovery). All measures of DNA damage were significantly elevated in all experimental groups for all doses studied. The average comet moment arm increased from 8.02 ± 0.62 in the control group to 13.14 ± 0.60 in the 0.1 LD_{50} group, 13.97 ± 0.54 in the 0.2 LD_{50} group, and 15.54 ± 0.54 in the 0.4 LD_{50} group. The average percentage of DNA fragmentation increased from 1.38 ± 0.14 in the 0.1 LD_{50} group, 2.68 ± 0.19 in the 0.2 LD_{50} group, and 3.30 ± 0.27 in the 0.4 LD_{50} group. The average comet tail length increased from 20.48 in the control group, to 35.03 ± 1.72 in the 0.1 LD_{50} group, 37.42 ± 1.64 in the 0.2 LD_{50} group, and 41.80 ± 1.61 in the 0.4 LD_{50} group.

Discussion

The results of the present study suggest that comet assays, or other measures of apoptosis or DNA damage in blood leukocytes, may provide sensitive biomarkers for assessment of cellular damage associated with low-dose CWNA exposure. Indeed, in the present study, increased DNA fragmentation could be detected in blood leukocytes over 2 weeks after exposure to even the lowest dose of soman used in this study, which did not elicit overt pathology. While specificity for any one type of toxic exposure over another cannot be made without the completion of additional experiments studying other toxic compounds, our results do indicate that comet assays measuring DNA damage may provide a rapid and relatively simple blood screen to determine if subclinical pathology is present.

Organophosphates such as soman and sarin exert their toxic effects primarily through a potent inhibitory action on acetylcholinesterase, the enzyme that degrades the neurotransmitter acetylcholine. This leads to increased concentrations of acetylcholine in the brain and at neuromuscular junctions, resulting in epileptic seizures and muscle tetany. Soman (methylphosphonofluoridic acid 1,2,2-trimethylpropyl ester) is a potent acetylcholinesterase inhibitor [1]. The neuropathological sequelae of severe soman toxicity include neural lesions of the amygdala, hippocampus, piriform cortex and thalamus [6]. However, the effects of organophosphate poisoning on the immune system have been less well characterized.

Sub-lethal doses of soman resulted in significant increases in all measures of leukocyte DNA fragmentation, at all doses and time points examined, as compared with saline-injected control animals. While these results demonstrate moderately increased leukocyte apoptosis following low-dose soman exposure, the mechanism by which apoptosis is elicited is unknown. Similarly, the pathological and/or immune consequences of small, but statistically significant increases in leukocyte apoptosis following soman exposure are unknown. No clear dose-response effect on DNA fragmentation was observed using the comet method, but direct cell counts demonstrated a dose-response relationship to the percentage of apoptotic cells. This finding suggests that among the affected leukocytes in each experimental condition, the degree or amount of DNA damage was similar. However, the percentage of affected leukocytes was directly related to the dose of soman. An unexpected finding in the present study was the long-term nature of the DNA damage associated with low-level soman exposure. Significantly, increased measures of apoptosis were observed after 17 days of recovery after the last exposure to the toxicant. Long-term reductions in leukocyte responsiveness have been reported after low-level exposure to another CWNA, sarin [7], but the mechanism by which such long-term leukocytic damage occurs is not known. Considering the long-term nature of the damage, organophosphates may damage at the level of stem cells in the bone marrow, which could then lead to increased apoptosis in the derived leukocytes.

While the link between organophosphate poisoning and leukocyte apoptosis remains uncertain, data suggest that reactive oxygen molecules may be involved in neuronal apoptosis after exposure to the cholinesterase inhibitor pyridostigmine bromide [8]. Release of reactive oxygen species after pyridostigmine bromide treatment was found to be mediated by muscarinic acetylcholine receptors and NMDA glutamate receptors, and pretreatment with atropine or MK-801 blocked reactive oxygen species generation. Similar mechanisms could possibly be involved in the leukocyte damage observed in the present study after soman exposure. If confirmed in the case of nerve agent exposure, current organophosphate poisoning treatments may benefit by cotreatment with antioxidant compounds, and inhibitors of reactive oxygen species generation.

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