Dehydroepiandrosterone (DHEA) Sulfate Prevents Reduction in Tissue Vitamin E and Increased Lipid Peroxidation Due to Murine Retrovirus Infection of Aged Mice

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Abstract. **Dietary effects of dehydroepiandrosterone sulfate (DHEAS) supplementation on tissue antioxidants and lipids were investigated in retrovirus infected mice. DHEA is a powerful antioxidant and immunomodulator whose production declines with age. For this study, twenty-four female, 15-month-old C57BL/6 mice were left uninfected while twenty-four were infected with LP-BM5 murine leukemia virus, causing murine AIDS. The retroviral infection caused immune dysfunction and loss of hepatic and cardiac vitamins E and A, resulting in increased lipid peroxides. Treatment with DHEAS at 0.01 or 0.005% in drinking water for 10 weeks post-infection significantly ($P < 0.05$) lowered lipid peroxidation in both heart and liver tissues. Treatment with DHEAS also largely prevented loss of the antioxidants, such as vitamin E and A, and prevented loss of phospholipid in the hearts and livers of the old uninfected as well as infected mice. This study suggests that DHEAS supplementation reduces damage associated with elevated oxidation due to aging and retrovirus infection.** [P.S.E.B.M. 1998, Vol 218]

Dehydroepiandrosterone sulfate (DHEAS) is an adrenal hormone whose low levels are related to aging, greater incidence of various cancers, immune dysfunction, atherosclerosis, and osteoporosis (1). As these conditions frequently increase free radical damage, the antioxidant activity of DHEAS may help to prevent oxidation-induced diseases (2). DHEAS levels in the blood also decline with progression of human immunodeficiency virus (HIV) infection (3). DHEAS is rapidly converted into lipoidal derivatives such as DHEA sulfide, a more potent inhibitor of glucose-6-phosphate dehydrogenase than DHEA (4). This is a possible mechanism for the inhibition of farnesyl transferase in the cholesterol pathway that promotes *ras* oncogene protein (P^{21} *ras*) in plasma membranes (5).

Murine acquired immune deficiency syndrome (AIDS) is strikingly similar to human AIDS, even though the infecting agent, HIV, and LP-BM5 murine leukemia virus are different types of retroviruses (6). Murine and human AIDS are characterized by lymphadenopathy, splenomegaly, reduced T cell function, loss of disease resistance, oxidative damage, and tissue vitamin E and A deficiency (7–9).

Superoxide radicals, hydrogen peroxides, hydroxyl radicals, and other lipid peroxides are produced at high levels when immune defenses are suppressed. Then increasing exposure to bacterial toxins and mitogens stimulates macrophages to produce free radicals. These molecules should facilitate disease progression from HIV infection to AIDS (10) by reacting with immunomodulatory antioxidants, inducing their deficiency, and thus accelerating pathogenesis. Vitamin E is an important immune modulator

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Supported as model for human leukemia by grants from Phi Beta Psi, Wallace Genetics Foundation, Inc and by NIH, HL59794.

Received September 12, 1997. [P.S.E.B.M. 1998, Vol 218]
Accepted February 5, 1998.

0037-9727/98/2183-0210$10.50/0
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in AIDS (11), as it reduces free radicals during development of immune dysfunction (12). Vitamin E supplementation slowed immunosuppression and reduced lipid peroxides (13), retarding the development of MAIDS. DHEAS treatment largely prevented the loss of immune function during murine retroviral infection (14) and aging (15). In this study, dietary supplementation with different doses of DHEAS was investigated to observe the effects upon the increased oxidation associated with aging and retrovirus infection. The current study determined that DHEAS supplementation prevents oxidative damage and vitamin E deficiency in old as well as retrovirus-infected old C57BL/mice. It also modified lipid levels, which affected cardiovascular disease risk.

Methods and Materials

Reagents. Chemicals including DHEAS, methanol, chloroform, standards for lipids, and KOH were purchased from Sigma Chemical Co. (St. Louis, MO), and vitamin E and vitamin A standards were obtained from Eastman Chemical Co. (Rochester NY) unless stated.

Animals and Murine AIDS. Female, 15-month-old C57BL/6 mice were obtained from the Charles River Laboratories Inc. (Wilmington, DE). They were housed four mice per transparent plastic cage with a stainless steel wire lid. They were kept in the animal facility of the Arizona Health Sciences Center. The University of Arizona Committee on Animal Research approved the study protocol. The housing facility was maintained at 20°–22°C and 60%–80% relative humidity, with a 12-hr light:dark cycle. Water and diet (Harlan Diet #7002, Madison, WI) were freely available. The diet contained 0.64 mg of d-α-tocopherol/kg of diet. Food intake, fluid intake, and body weight were measured every two days. Dehydroepiandrosterone sulfate (DHEAS) was dissolved in tap water and made available to the mice in a 100-ml graduated plastic cylinder with a stopper. Half of the mice were infected on Day 0. The LP-BM5 murine leukemia retrovirus mixture was administered intraperitoneally to mice in 0.1 ml minimum essential medium (MEM) with an esotropic titer (XC) of 4.5 $\log_{10}$ plaque forming units × 10^{-3}/l, which induces disease as previously described (6). Uninfected mice were injected with 0.1 ml MEM and used as controls. Infection of an adult female C57BL/6 mouse with the retrovirus leads to the rapid induction of clinical symptoms without any latent phase (6). After 2 weeks of housing, eight mice were randomly assigned to six treatment groups. The groups were: uninfected mice, or LP-BM5 infected mice given unsupplemented tap water; LP-BM5–infected or uninfected mice that consumed about 0.44 μg of DHEAS/mouse/day in their drinking water (0.01% of the water was DHEAS); infected and uninfected mice that consumed about 0.22 μg DHEAS/mouse/day in their drinking water (0.005% of the water was DHEAS). The infection and treatment period were 10 weeks for all groups. Mice were sacrificed while under ether anesthesia as described previously (12) using a procedure approved by University of Arizona Animal Care. The subclavian blood, liver, heart, mesenteric lymph nodes, and skin samples were collected aseptically and stored at −70°C for future analysis.

Assays

Measurement of DHEAS. Dehydroepiandrosterone sulfate (DHEAS) in serum was measured by radioimmunoassay (RIA) ^{125}I-DHEA-SO_4, Cota-A-Count DHEA sulfate (Diagnostic Products Corporation, Los Angeles, CA). Briefly, 50 μl of samples were placed in DHEA-SO_4 Ab-coated tubes and 1.0 ml of the tracer ^{125}I-DHEA-SO_4 was dispensed into the tubes within 10 min. Then, the tubes were incubated for 30 min at 37°C in a water bath. The contents of all tubes was decanted, and the tubes were allowed to drain for 2 or 3 min. Then the tubes were stroked sharply on absorbent paper to shake off all residual droplets. A gamma counter determined radioactivity. Data were presented as counts/min (cpm), and DHEAS concentration was calculated from a log-log representation of the calibration curve.

Determination of Conjugated Dienes and Lipid Fluorescence. Approximately 0.2 g of tissue was homogenized in 5.0 ml of Folch solution, 2:1 v/v chloroform: methanol (16). After protein separation, a 0.1-ml fraction was dried in a steady flow of nitrogen gas at 55°C. Then 2 ml of chloroform were added and analyzed for conjugated dienes and lipid fluorescence as previously described (17, 18). The conjugated diene fatty acids were determined by obtaining the absorbency of each sample solution at 237 nm (Shimadzo UV 160 UV recording spectrophotometer Tokyo, Japan). Lipid fluorescence was measured in an Aminco Bowman fluorescence spectrophotometer (Baltimore, USA). Maximum fluorescence at 470 nm was measured. The activation wavelength was set at 395 nm.

Determinations of Phospholipid. The phospholipid contents of the livers and hearts were determined by the method of Raheja *et al.* (19). This method does not require the predigestion of the phospholipid. Briefly 0.3 ml of Folch extract solution was dried under air at 75°C and then 0.5 ml of chloroform was added. Then 0.2 ml of a coloring reagent was added, mixed well and placed in a boiling water bath for 1.5 min. Then 3.0 ml of CCl_4 were added. The phospholipid level was determined by obtaining absorbency at 710 nm in a spectrophotometer using appropriate blanks and standards. Dipalmitoyl phosphatidylcholine was used to produce the standard curve.

Determination of Total Cholesterol. The total cholesterol of the liver and heart was determined by the method of Zak (20). Briefly 0.3 ml of Folch extract was dried under air at 70°C. Then 3 ml of Zak's reagent was added and mixed thoroughly with 2 ml of sulfuric acid. Total cholesterol was determined by measuring the absorbency at 560 nm in a spectrophotometer using appropriate blanks and standards.

Determination of Triglycerides. The triglycerides of the liver and hearts were determined by the method of

Brigges *et al.* (21). Briefly, 50.0 μl of Folch extract in a 15-ml test tube was dried under air at 70°C and then mixed thoroughly with 0.1 ml of 100% ethanol. Then 1.0 ml of TG-GPO-trinder 10 was added to each sample. Triglycerides were determined by comparing the absorbency at 540 nm in a spectrophotometer with appropriate standards of triheptadecanoin.

Determination of Vitamin E and A. Vitamin E and retinol (vitamin A) in liver and heart tissues were determined by the fluorometric method described by Dugan *et al.* (22). Briefly, approximately 0.2 g of tissue was homogenized in 5.0 ml of Folch extract. Then 0.3 ml of the above Folch extract was dried under N_2 gas followed by adding 1.0 ml of ethanol and 0.5 ml of 25% ascorbic acid. After 1 ml of 10N KOH was added to the solution, the mixture was incubated for 35 min at 70°C, and 5 ml of n-hexane was added. To determine the vitamin A level, 2.0 ml of the n-hexane layer were removed and measured at an emission of 430 nm and an excitation of 365 nm in a fluorometer using an appropriate blank reagent. Then the 2.0-ml n-hexane layer was added back into the original solution. To that solution, 0.6 ml of (10N) 60% sulfuric acid was added, and the mixture was vortexed for 30 sec. It was centrifuged at 1000 rpm for 10 min. Then the fluorescence intensity of vitamin E was determined at an emission of 340 nm and or excitation maximum of 295 nm. External standards of d-alpha-tocopherol and all-trans-retinol were used for preparing standard curves. All vitamin levels were measured by μg/g wet tissue.

Statistical Analysis. All results are expressed as the means ± SEM. Data of lipid analysis and content of serum DHEAS were analyzed. When data for more than two means were compared, the differences between means were analyzed by ANOVA and determined by Duncan's multiple range test. A value of $P < 0.05$ was considered significant.

Results

Effects of DHEAS Supplementation on the Body and Spleen Weight, and Serum DHEAS. Body weight was not affected by retrovirus infection or by DHEAS consumption. Also, fluid and diet intakes remained constant throughout the investigation (data not shown). Spleen weights (10 weeks postinfection) were elevated and significantly ($P < 0.05$) greater, about 6-fold higher, in the infected mice than in the uninfected controls. Spleens from uninfected mice weighed 0.122 ± 0.03 mg, significantly ($P < 0.05$) less than those of infected mice (0.61 ± 0.6 mg). This indicates that the infection progressed toward murine AIDS. DHEAS serum levels were significantly ($P < 0.05$) elevated by DHEAS treatments of 0.22 μg (0.005% DHEAS) and 0.44 μg (0.01% DHEAS) of DHEA/mouse/day (Table I).

The Cardiac and Hepatic Vitamin E and A Levels. Hepatic and cardiac vitamin E levels (Fig. 1) were significantly ($P < 0.05$) reduced by retrovirus infection as found previously in young mice (12). DHEAS treatment at 0.22 and 0.44 μg/mouse/day significantly ($P < 0.05$) retarded the loss of tissue vitamin A and E during infection. Infected old mice, treated with the higher DHEAS supplement, maintained hepatic and cardiac levels of vitamin E (Fig. 1) and A (Fig. 2) similar to those of uninfected, untreated old mice. Uninfected mice fed DHEAS showed a large increase ($P < 0.05$) in hepatic and cardiac vitamin E and A compared to untreated old mice (Figs. 1, 2).

The Cardiac and Hepatic Lipid Peroxidation. Retrovirus infection significantly ($P < 0.05$) increased hepatic and cardiac free radical reaction products: lipid fluorescence and diene conjugates (Figs. 3, 4). DHEAS treatment significantly ($P < 0.05$) prevented increased hepatic and cardiac lipid fluoresence and diene conjugates (Figs. 3, 4). There was a dose effect relationship as both infected and uninfected old mice treated with 0.44 μg DHEAS/mouse/day had significantly ($P < 0.05$) lower conjugated dienes and lipid fluorescence than old mice fed 0.22 μg DHEAS/mouse day of DHEAS (Figs. 3, 4).

The Cardiac and Hepatic Total Cholesterol. At 10 weeks postinfection, mice showed an increase in total cholesterol levels ($P < 0.05$) in the heart and liver. DHEAS supplementation of uninfected old mice did not cause any significant change in total cholesterol (Tables II, III).

Table I. Intake of DHEAS and Water with Modification of Serum DHEAS

Treatments		Consumed	Actual
DHEAS (%)	Retrovirus infection	Water /day/mouse (ml)	Serum DHEAS (μg/dl)
None	without	6.8 ± 0.5	0.9 ± 0.6
None	with	6.0 ± 0.4	0.7 ± 0.2
0.005	without	6.1 ± 0.4	*11.6 ± 0.22
0.005	with	5.9 ± 0.4	*4.9 ± 0.75
0.01	without	6.3 ± 0.5	*19.7 ± 0.62
0.01	with	5.7 ± 0.6	*11.8 ± 0.53

Note. Results are mean of eight observations in amounts of DHEAS and water consumption. The serum DHEAS are duplicate readings from 4 mice per group.
* $P < 0.05$ significantly different from infected mice in same treatment group and untreated groups.
n = 8 mice per group, SE: Standard Error.

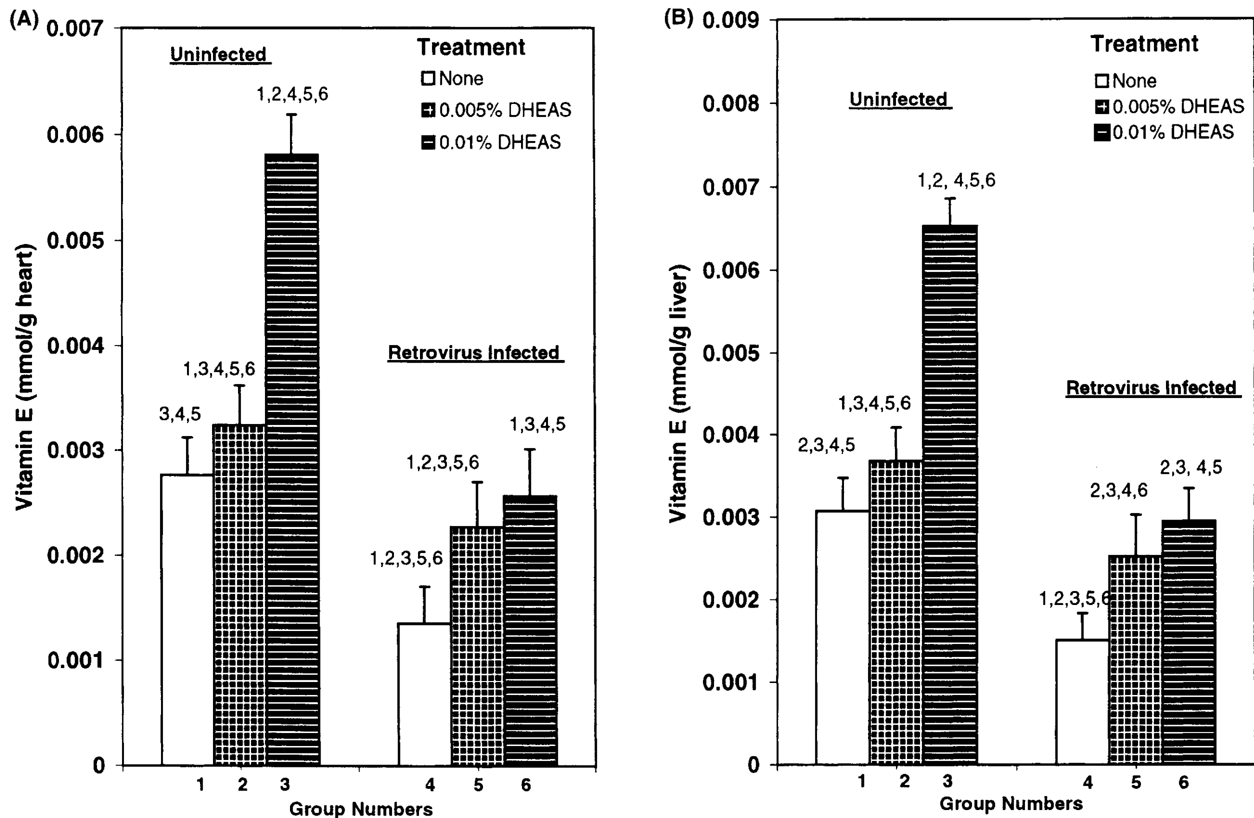

Figure 1. Modification of cardiac (left panel) and hepatic (right panel) vitamin E levels in uninfected and infected old mice by DHEAS. The data from two different DHEAS concentrations are compared among groups. Results are mean ± SE, $n = 8$. For each dose of DHEAS, values with different letters above the bar are significantly different $^{*}P < 0.05$ as determined by Duncan's Multiple Range. Each group was given a number. Numbers above a bar show that numbered groups are different from it.

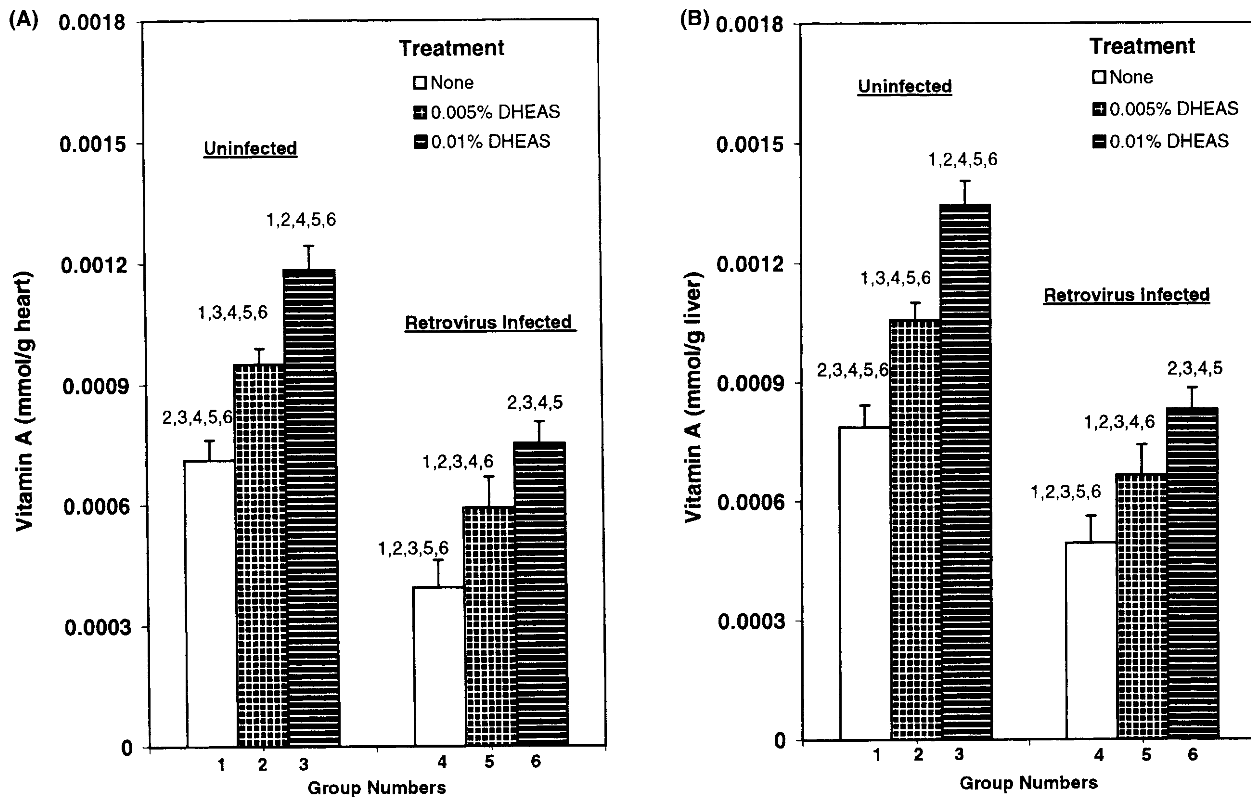

Figure 2. Modification of cardiac (left panel) and hepatic (right panel) vitamin A levels in uninfected and infected old mice by DHEAS. The data from two different DHEAS concentrations are compared among groups. Results are mean ± SE, $n = 8$. For each dose of DHEAS, values with different letters above the bar are significantly different $^{*}P < 0.05$ as determined by Duncan's Multiple Range. Each group was given a number. Numbers above a bar show that numbered groups are different from it.

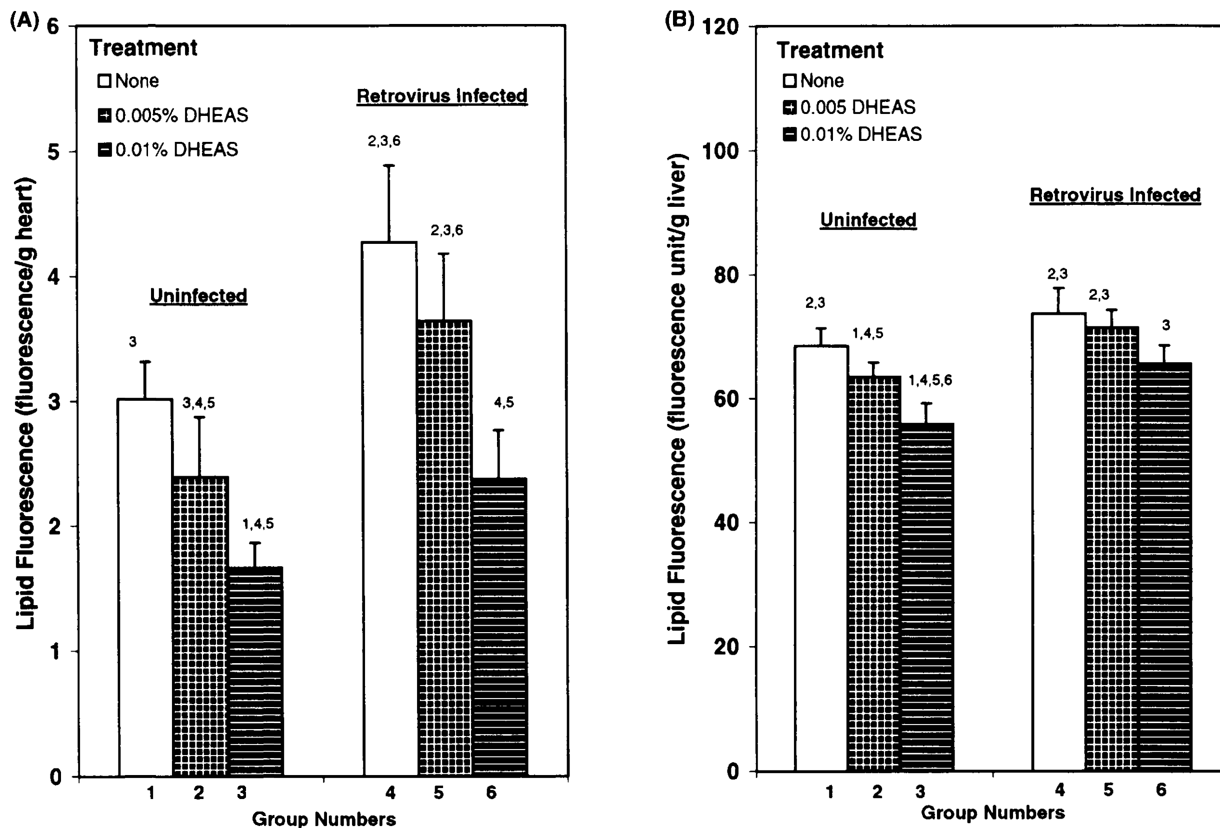

Figure 3. Modification of cardiac (left panel) and hepatic (right panel) lipid fluorescence levels in uninfected and infected old mice by DHEAS. The data from two different DHEAS concentrations are compared among groups. Results are mean ± SE, $n = 8$. For each dose of DHEAS, values with different letters above the bar are significantly different $^*P < 0.05$ as determined by Duncan's Multiple Range. Each group was given a number. Numbers above a bar show that numbered groups are different from it.

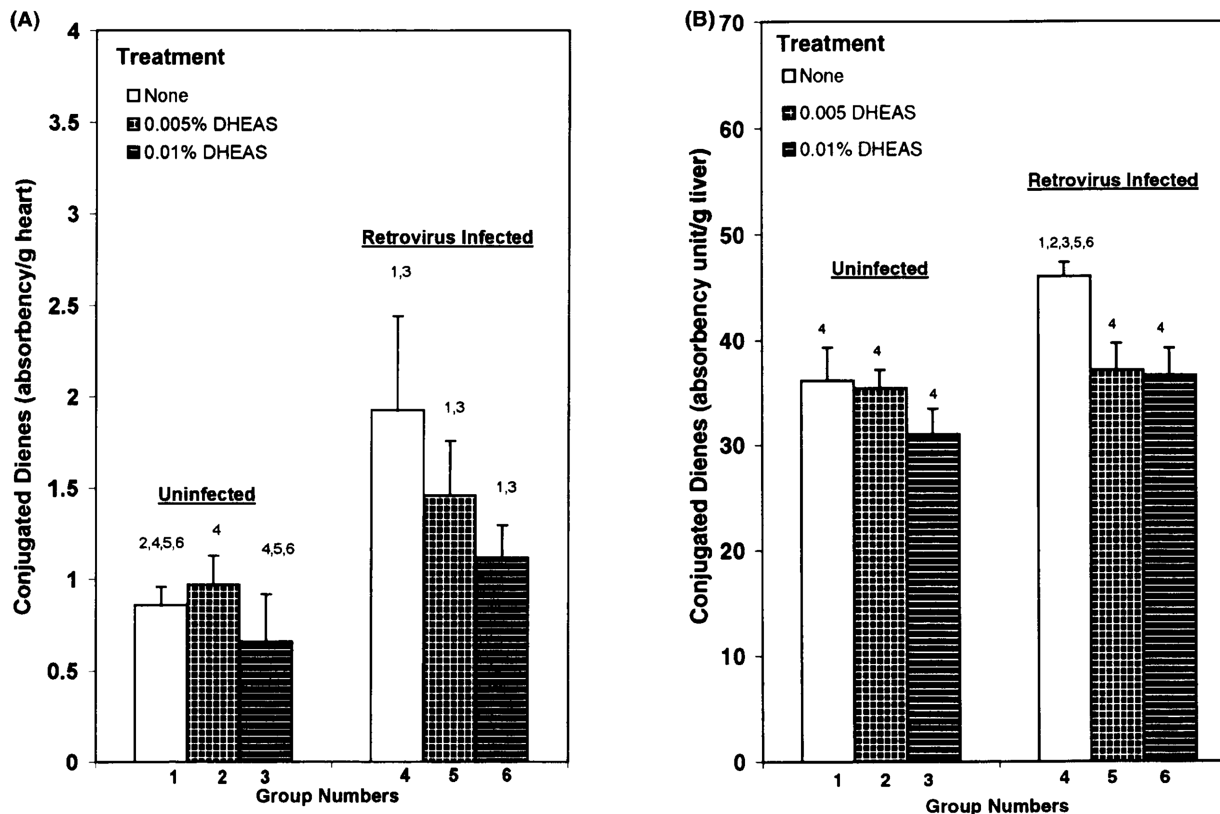

Figure 4. Modification of cardiac (left panel) and hepatic (right panel) conjugated diene levels in uninfected and infected old mice by DHEAS. The data from two different DHEAS concentrations are compared among groups. Results are mean ± SE, $n = 8$. For each dose of DHEAS, values with different letters above the bar are significantly different $^*P < 0.05$ as determined by Duncan's Multiple Range. Each group was given a number. Numbers above a bar show that numbered groups are different from it.

Table II. Modification of Cardiac Lipid Profile by DHEAS, Aged and Retrovirus Infection

Treatments: DHEAS	Retrovirus infection	Triglycerides (m mol/g tissue)	Phospholipid (m mol/g tissue)	Total cholesterol (m mol/g tissue)	Cholesterol/phospholipid (mol/mol ratio)
None	–	*0.043 ± 0.005	*0.176 ± 0.002	*0.017 ± 0.0038	*0.05 ± 0.01
None	+	*0.054 ± 0.01	0.14 ± 0.02	0.024 + 0.0006	0.07 ± 0.02
0.005%	–	*0.04 ± .002	*0.204 ± 0.033	*0.016 + 0.0015	*0.05 + 0.02
0.005%	+	*0.046 ± 0.0008	*0.16 ± 0.02	*0.017 + 0.0003	*0.05 ± 0.01
0.01%	–	*0.04 ± 0.003	*0.22 ± .023	*0.0139 ± 0.0006	*0.03 ± 0.02
0.01%	+	*0.04 ± 0.0023	*0.187 ± 0.02	*0.016 ± 0.0008	*0.04 ± 0.02

Notes. Values are means ± SE; $n = 8$
Abbreviations used: DHEAS, dehydroepiandrosterone sulfate; – uninfected; + retrovirus infected.
* $P < 0.05$ significantly different from infected mice.

Table III. Modification of Hepatic Lipid Profile by DHEAS, Aged and Retrovirus Infection

Treatments: DHEA	Retrovirus infection	Triglycerides (m mol/g tissue)	Phospholipid (m mol/g tissue)	Total cholesterol (m mol/g tissue)	Cholesterol/phospholipid (mol/mol ratio)
None	–	*0.088 ± 0.007	*0.41 ± 0.005	*0.017 ± 0.005	*0.415 ± 0.06
None	+	0.107 ± 0.012	0.03 ± 0.006	0.045 ± 0.0015	1.2 ± 0.04
0.005%	–	*0.0804 ± 0.005	*0.041 ± 0.008	*0.016 ± 0.007	*0.39 ± 0.03
0.005%	+	*0.092 ± 0.088	*0.04 ± 0.006	*0.019 ± 0.001	*0.47 ± 0.04
0.01%	–	*0.076 ± 0.001	0.047 ± 0.002	*0.016 ± 0.001	*0.343 ± 0.06
0.01%	+	*0.089 ± 0.008	*0.044 ± 0.009	*0.018 ± 0.002	*0.422 ± 0.07

Note. Values are means ± SE; $n = 8$
Abbreviations used: DHEAS, dehydroepiandrosterone sulfate; – uninfected; +retrovirus infected.
* $P < 0.05$, significantly different from infected mice.

DHEAS significantly ($P < 0.05$) maintained total cholesterol levels similar to those of uninfected mice.

The Cardiac and Hepatic Triglycerides. Retrovirus infection significantly ($P < 0.05$) increased hepatic and cardiac triglycerides (Tables II, III). DHEAS treatment reduced hepatic and cardiac triglycerides in uninfected as well as in infected old mice (Tables II, III). 0.01% DHEAS consumption produced significantly fewer triglycerides in infected mice than did 0.05% DHEAS.

The Cardiac and Hepatic Total Cholesterol/Phospholipid Ratio. Retrovirus infection significantly ($P < 0.05$) increased cholesterol:phospholipid ratios in cardiac (Table II) but not hepatic tissues (Table III). DHEAS treatment begun 2 weeks postinfection significantly ($P < 0.05$) prevented increases in cardiac cholesterol:phospholipid ratios (Table II). The old mice treated with 0.01% DHEAS showed a significant ($P < 0.05$) decline in cardiac cholesterol:phospholipid ratio compared to levels found in unsupplemented, infected old mice (Tables II, III).

The Cardiac and Hepatic Phospholipids. Retrovirus infection significantly ($P < 0.05$) lowered phospholipid levels in cardiac and hepatic tissues (Tables II, III). Infected and uninfected old mice supplemented with the higher DHEA dose showed significantly ($P < 0.05$) higher phospholipid levels in both cardiac and hepatic tissues (Tables II, III).

Discussion

Retrovirus infection in old mice caused a severe loss of hepatic vitamin E and A, along with a significant increase in conjugated dienes, lipid fluorescence, and triglycerides (11). This is similar to changes caused by HIV infection in humans (11, 23). We found that cardiac and hepatic conjugated dienes, lipid fluorescence, triglycerides, and cholesterol levels were significantly lower after DHEAS supplementation in uninfected as well as infected old mice. Thus, DHEAS treatment substantially reduced the oxidative stress due to age and/or murine retrovirus infection. Vitamin E and A losses were also prevented by DHEAS consumption in uninfected old mice. Such changes should reduce (11) some of their cardiovascular disease risk factors, increased lipids and lipid peroxides.

In vitro studies have shown that DHEA had more antioxidant activity than vitamin E, the biological molecule with the presumed highest antioxidant activities (24). All groups of mice consumed approximately 6.0 g/day of the diet with 0.40 mg/day of d-α-tocopherol. Therefore, the amount of dietary antioxidants consumed, vitamin E or DHEAS, increased to 50% and 100% in the DHEAS supplemented mice. A 150%–450% increase in dietary vitamin E only partially prevented the increased oxidation in retrovirus-infected mice (12). Moreover, hepatic vitamin E was about 40% lower in retrovirus-infected mice (12). More-

over, hepatic vitamin E was about 40% lower in retrovirus-infected, old mice than in infected young mice and 250% lower than in uninfected, young mice (12). Therefore, DHEAS supplementation in mice may stimulate enzymes that reduce oxidized-antioxidants, increase production of glutathione activity, and/or prevent vitamin E loss (1).

DHEAS reduced the increased lipid peroxidation associated with aging and murine AIDS (11). Consequently, increased DHEAS modified changes in lipid levels, vitamins E and A, and oxidative stress of cardiac and hepatic tissues otherwise caused by aging and/or retroviral infection (12, 25). Thus it should increase cellular function that otherwise would be destroyed by free radical activity. Not surprisingly, impaired immune function in aged or retrovirus-infected mice was partly restored by DHEAS (14, 15).

Increases in lipid peroxidation during aging could modify membranes by cross linking fatty acids with the proteins or other lipids to reduce membrane fluidity (25). Malonadialdehyde alters membrane structure and function by cross-linking with amino groups. In addition 4-hydroxynonenal reacted with thiol compounds causing a rapid loss of their reducing power and thus inhibiting DNA and protein synthesis (25). DHEA did not prevent the loss of enzymes that are impaired by carbon tetrachloride that cause liver damage; rather it reduced lipid peroxidation (26). DHEAS, the major form of DHEA, prevented loss of glucose-6-phosphatase and gamma-glutamyltranspeptidase that are sensitive to peroxidation (27). We found that the DHEAS-supplemented old mice maintained vitamin E levels while reducing oxidative damage. As DHEAS levels decline with age, prevention of vitamin E and A loss may delay the aging process as well as slow the progression towards AIDS (28, 11, 12). Since rapidly proliferating cells of the immune system are susceptible to oxidative damage by peroxides and superoxides, immune enhancement by vitamin E is expected (11). Opportunistic pathogens increase as the immune system is dysregulated in murine AIDS and aging (6, 1). The pathogens stimulate phagocytes to produce free radicals, increasing lipid peroxidation, and consequently, loss of vitamin E (29). Immune dysfunction induced by the retrovirus (8, 12) as well as age (15) alters cytokine production, vital for immune regulation. The retrovirus infection increases oxidative stress while decreasing tissue antioxidants (13). DHEAS prevented immune dysfunction (14) and this would be expected to prevent oxidation damage and loss of vitamin E.

Reduced vitamin A and E levels in patients infected with HIV enhance retroviral replication by protein kinase C (30, 7). The HIV coat protein Gp 120 induces protein kinase C activity in lymphocytes. This should result in transition from HIV latency to active replication in lymphocytes with upregulation of HIV replication in cells. Redox-regulated HIV transcription is inhibited by antioxidants that replenish intracellular glutathione (29). Thus, prevention of oxidative damage associated with murine AIDS should slow retrovirus development by preventing its action as well as its stimulation of immune dysfunction (11, 7). Reduced tissue levels of vitamin E were not due to a lower intake during murine AIDS as food consumption was unchanged (12). During murine retrovirus infection, supplementation with high levels of vitamin E restored tissue vitamins E and A while it partially normalized immune dysfunction (12). Our current studies demonstrated, for the first time, that treatment with DHEAS significantly decreased oxidative stress associated with murine retrovirus infection while maintaining tissue vitamin E and A levels.

Free radicals induce expression of HIV in human T cell lines by starting transcription of NF-KB (nuclear factor-k8). Maintenance of tissue vitamin E by DHEAS supplementation should block NF-kB activation by reducing oxidative stress, thereby inhibiting HIV replication (32). Reduced levels of antioxidants, including vitamin E and DHEAS, correlated well with accelerated progression to human AIDS (11, 3, 30). Oxidative stress may also be a potent inducer of viral activation by causing DNA damage in infected cells. Increased superoxide dismutase activity in murine AIDS results in higher hydrogen peroxide production, more oxidative damage, and loss of vitamin E. Plasma DHEAS declines with age while the incidence of cardiovascular disease and cancer increases (1). Although all individuals aged 50–79 years had greatly reduced DHEAS, those with the highest DHEAS levels had the lowest risk for cardiovascular disease (31). Elevated plasma triglycerides and cholesterol levels during aging declined after treatment with DHEAS. In summary, DHEAS supplementation provides protection against pro-oxidants and prevents lipid profile changes induced by aging and retrovirus infection.

We wish to acknowledge Dr. GH Atkinson from the Department of Biochemistry for his assistance in lipid analysis.

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