Effect of Dehydroepiandrosterone and its Sulfate Metabolite on Neuronal Cell Viability in Culture

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Abstract

Background: The neurosteroids dehydroepiandrosterone (DHEA) and its sulfated metabolite (DHEAS) have been reported to possess neuroprotective as well as anti-tumoral activity *in vitro* and *in vivo*.

Objectives: To compare the effect of the two neurohormones on cell viability in primary whole-brain fetal mouse culture and isolated neuronal culture, as well as in a human neuroblastoma cell line (SK-N-SH).

Methods: Cell viability and cell proliferation were determined with the neutral red and ^{3}H-thymidine uptake methods. Apoptosis in propidium iodide-stained neuroblastoma cells was determined using flow cytometry.

Results: DHEA (1 nM–10 μM) decreased the viability of selected primary neuronal cells (33–95% after 24 and 72 hours) but not of whole-brain cultured cells (neuron + glia). DHEAS did not significantly modify cell viability in either primary culture. In a human neuroblastoma cell line, DHEA (1 nM–1 μM) decreased ^{3}H-thymidine uptake (30–60%) and cell viability (23–52%) after 24 hours. DHEAS did not significantly modify, or only slightly stimulated, cell viability and uptake of ^{3}H-thymidine (132% of controls). The combination of DHEA and DHEAS neutralized the toxic effect of DHEA in both primary neuronal culture and neuroblastoma cell line. Flow cytometric analysis of DNA fragmentation in neuroblastoma cells treated with 100 nM DHEA/DHEAS for 24 hours showed an increase in apoptotic events (31.9% and 26.3%, respectively, vs. control 2.54%).

Conclusions: Our results do not confirm a neuroprotective role for DHEA and suggest that DHEA and DHEAS have a differential role: DHEA possesses a neurotoxic (expressed only in isolated neurons) and anti-proliferative effect; DHEAS demonstrates only a slight neuroprotective effect.

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DHEA = dehydroepiandrosterone
DHEAS = DHEA sulfate

The steroid hormone dehydroepiandrosterone and its sulfated derivative are found at high concentrations in different areas of the brain as well as in the peripheral circulation [1,2]. In the central nervous system, these steroids are biosynthesized by differentiated oligodendrocytes and astrocytes [3]. DHEA and DHEAS are metabolically interconvertible, and the ratio of DHEA/DHEAS depends on sulfotransferase activity for conjugation, and sulfatase activity for hydrolysis [4]. Previous studies have suggested that these steroids facilitate neuronal and glial cell survival [5] and protect hippocampal neurons from the excitatory amino acid N-methyl-D-aspartate [6]. Experimental studies have shown that DHEA/S facilitated the survival of neuronal and glial cells *in vitro* and improved memory and learning in young and aged mice [7,8]. Plasma levels of DHEA/S are age-dependent, decreasing dramatically in the elderly [3,5]. Some clinical studies have suggested that supplementation of DHEA may offer benefits counteracting the deleterious effects of cardiovascular disease, cancer, immune suppression states, and Alzheimer's disease in the aging population [9–11]. However, more recent reports have failed to provide convincing evidence of DHEA's significant neuroprotective activity [12,13].

The aim of the present study was to determine the differential effect of DHEA and DHEAS on neuronal and glial cell viability and survival in primary brain tissue and in neuroblastoma cells.

Materials and Methods

Reagents

For tissue culture, minimum essential medium, horse serum, fetal calf serum, glucose, glutamine, gentamycin, Leibowitch L-15, DCCM and RPMI mediums were obtained from Beit HaEmek, Israel. Poly d-lysine, uridine, 5-fluorouridine, neutral red solution, dimethyl sulfoxide, DHEA and DHEAS were obtained from Sigma (St. Louis, MO, USA). The study was approved by the Rabin Medical Center Ethics Committee for Animal Experimentation.

Cell culture

- ***Primary brain culture.*** ICR mice (Harlan, Israel) at day 14–15 of pregnancy were used. Animals were anesthetized with ether and sacrificed. The embryos were obtained and their brains were removed and homogenized in medium containing glucose (0.6%), glutamine (0.2 mM) and gentamycin (75 μg/ml). Cells were distributed in wells (96-well microplate) previously dispensed with poly-d-lysine, 300,000–500,000 cells/well. Forty-eight hours later, 5-fluorodeoxyuridine + uridine was added to half the plates to obtain a selected neuronal culture [14]. Cells were treated with the different agents in triplicate for 24–72 hours. Cells treated with vehicle (MEM + HS + FCS + glutamine + gentamycin) served as controls.
- ***Neuroblastoma cell line.*** A human neuroblastoma cell line (type SK-N-SH) was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 μg/ml), streptomycin (100 μg/ml), nystatin (0.5 u/ml), and L-glutamine (2 mM). Confluent cultures were washed with phosphate-buffered saline, detached with 0.5 mM EDTA, centrifuged and subcultured in 96-well microtiter test plates. Cells were incubated under controlled conditions of 37°C, and 5% $C0_2$ / 95% air.
- ***^{3}H-thymidine incorporation.*** Neuroblastoma cells were placed in 96-well microtiter test plates ($2x10^4$ cells/well) and incubated with 1 μCi/ml ^{3}H-thymidine for 24 hours. Cells were then harvested and the incorporated radioactivity was determined by liquid scintillation counter.
- ***Cell viability.*** Cell viability in culture was determined by the neutral red method [15]. A solution of neutral red 1% in DCCM medium (which is absorbed by the lysosomes, coloring living cells) was added, and after fixation the cells were observed under a phase microscope and typical wells were photographed. The quantitative determination of cell viability was performed by washing excess reagent and eluting color from the cells using citrate buffer (0.02 M) in alcohol solution (50%). The intensity of the color was positively correlated to the number of viable cells. Colorimetric determination was performed in an enzyme-linked immunosorbent reader at 550 nM.
- ***Flow cytometry of neuroblastoma cells.*** Neuroblastoma cells were treated with 100 nM DHEA or DHEAS for 24 hours, then suspended using Dulbecco's PBS, centrifuged 10 min at 200 g. Cell pellets were gently resuspended in a citrate buffer containing 250 nM sucrose and transferred to polypropylene tubes. Cell nuclei were isolated and stained by the addition of trypsin (30 μg/ml) and propidium iodide fluorochrome (0.42 mg/ml). Prior to flow cytometry analysis the tubes were placed in the dark at 4°C. The fragmentation of nuclei was determined with a fluorescence-activated cell sorter (FACScan, Becton and Dickinson, CA, USA) equipped with an argon ion laser adjusted to an excitation wave length of 488 nm and with a doublet discrimination module. Red fluorescence was collected via a 610 nm-long pass filter. Lysis II (BD, Becton and Dickinson) software was used for data acquisition. Apoptotic nuclear changes were evaluated according to the criteria of Nicoletti et al. [16].

Statistical analysis

All experiments using DHEA and DHEAS were conducted in parallel. Each experiment was repeated at least twice. Unpaired two-tailed Student's *t*-test was used to compare the groups. Levels of $P<0.05$ were considered significant. Results concerning cell viability and thymidine incorporation were expressed as a percentage of controls, which were defined as 100% for each experiment.

Results

The effect of DHEA and DHEAS at concentrations ranging from 1 nM to 10 μM on selected neuronal cell viability (neutral red method) and on whole-brain (glia+neurons) cell viability in primary mouse embryo tissue is shown in Figure 1. In selected

PBS = phosphate-buffered saline

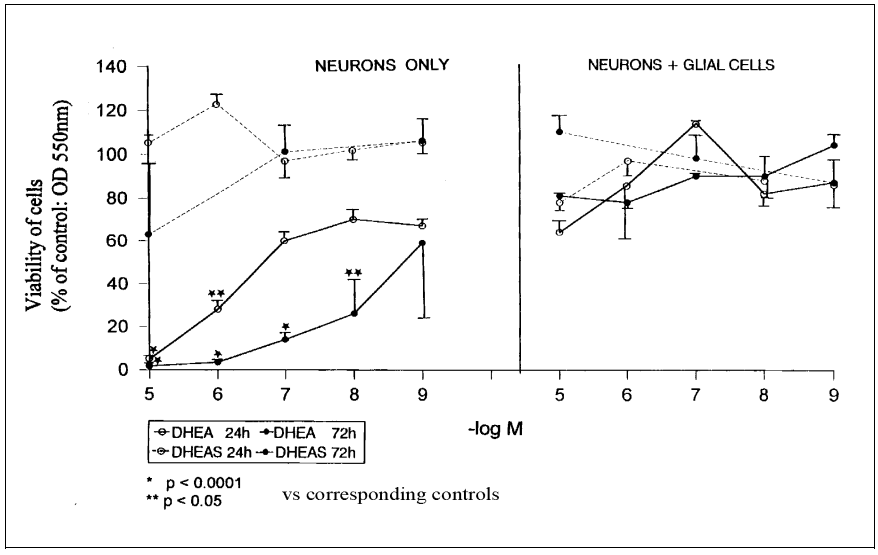

Figure 1. Dose (1 nM–10 μM) and time (24 and 72 hour) effect of DHEA/S on cell viability expressed as percent of control, in primary, mainly neuronal and whole-brain cell culture. Control cells were treated with vehicle and defined as 100%. Each point is the mean ± SEM of eight determinations.

MEM = mimimum essential medium
HS = horse serum
FCS = fetal calf serum

neuronal culture, DHEA induced a dose-dependent decrease in cell viability, compared to control (vehicle-treated) cells, leading to a 30–95% decrease after 24 or 72 hours. DHEAS did not significantly modify cell viability in either primary culture. Regression analysis of the dose-effect relationship yielded significance (r = 0.925, P<0.05) for both 24 and 72 hours. In contrast to the effect on selected neurons, in whole-brain culture (neurons+glia), cell viability was not significantly modified by the neurosteroids.

In neuroblastoma cells, DHEA (1 nM–1 μM) decreased ^{3}H-thymidine uptake by 32–68% (P<0.01 for 10 nM–1 μM) compared to untreated controls, whereas DHEAS did not significantly modify ^{3}H-thymidine uptake at either time point [Figure 2A]. Cell viability (neutral red method) in the neuroblastoma cell line significantly decreased (range 33–52%) (P<0.05) following treatment with DHEA (0.1–10 μM); DHEAS on the other hand did not significantly alter cell viability [Figure 2B].

The combination of the two neurohormones at a concentration of 100 nM completely antagonized the neurotoxic effect of DHEA alone (125% and 110% of control) after 24 and 48 hours, respectively [Figure 3].

Analysis of propidium iodide-stained neuroblastoma cells using flow cytometry is shown in Figure 4. In control cells, 97.4% of the events created a sharp, distinct peak of intact DNA, characteristic of the diploid form. Exposure to 100 nM DHEA or DHEAS induced a reduction in the intact DNA form and a shift to the left of the DNA peak, characteristic of fragmented nuclei, implying that 31.8% and 26.3% of the cells, respectively, were in the apoptotic state.

Discussion

The current enthusiasm for using DHEA as replacement therapy to counteract the effects of aging, and particularly for the treatment of Alzheimer's disease, is not upheld by scientific evidence. In one study, DHEA was found to increase, in PC12 cells, the production of the amyloid precursor protein, frequently associated with the pathology of Alzheimer's disease [17]. In

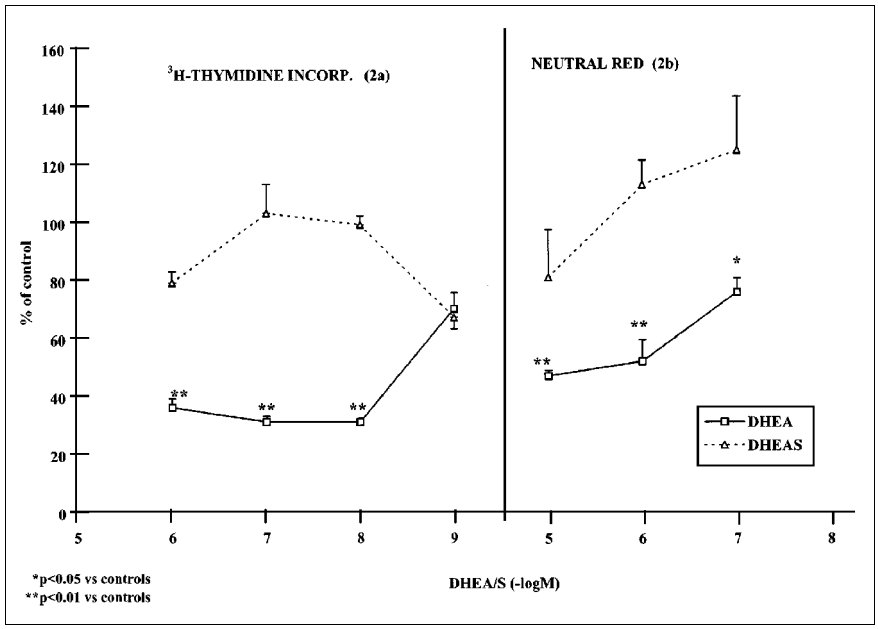

Figure 2. Effect of DHEA/S on cell proliferation using the ^{3}H-thymidine incorporation method **[A]** and on cell viability using neutral red method **[B]**, in neuroblastoma cell line 24 hours after hormone administration. Levels are expressed as percent of control (100%). Each point represents mean ± SEM of four to eight experiments.

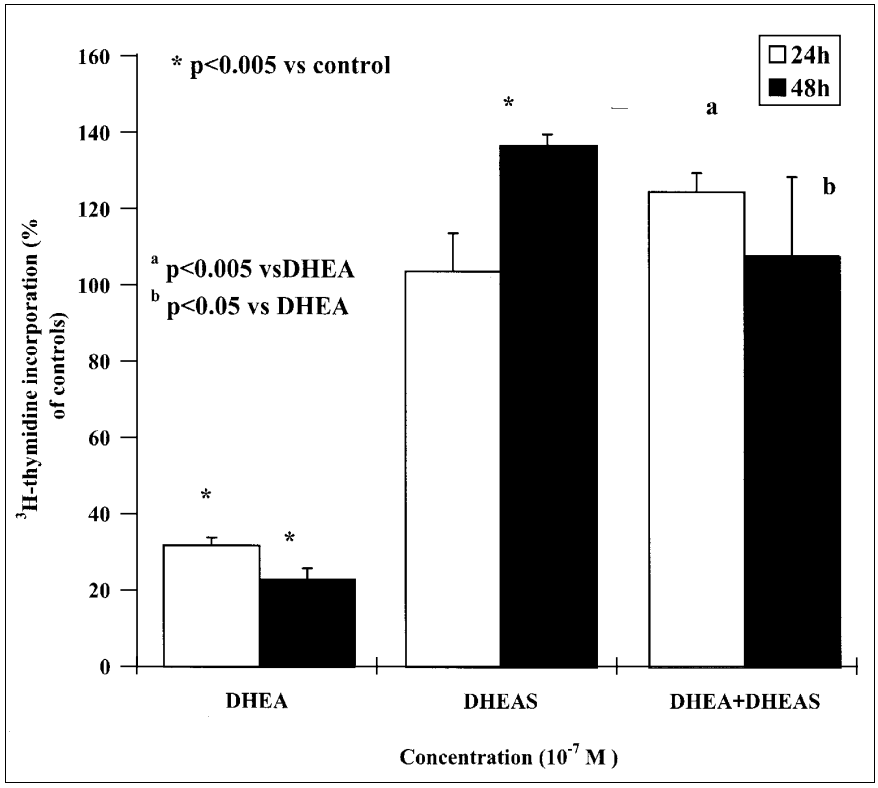

Figure 3. Effect of DHEA/S (100 nM) singly and combined on cell proliferation in neuroblastoma cell lines, using the ^{3}H-thymidine incorporation method. Levels are expressed as percent of control (100%). Each point represents mean ± SEM of four experiments.

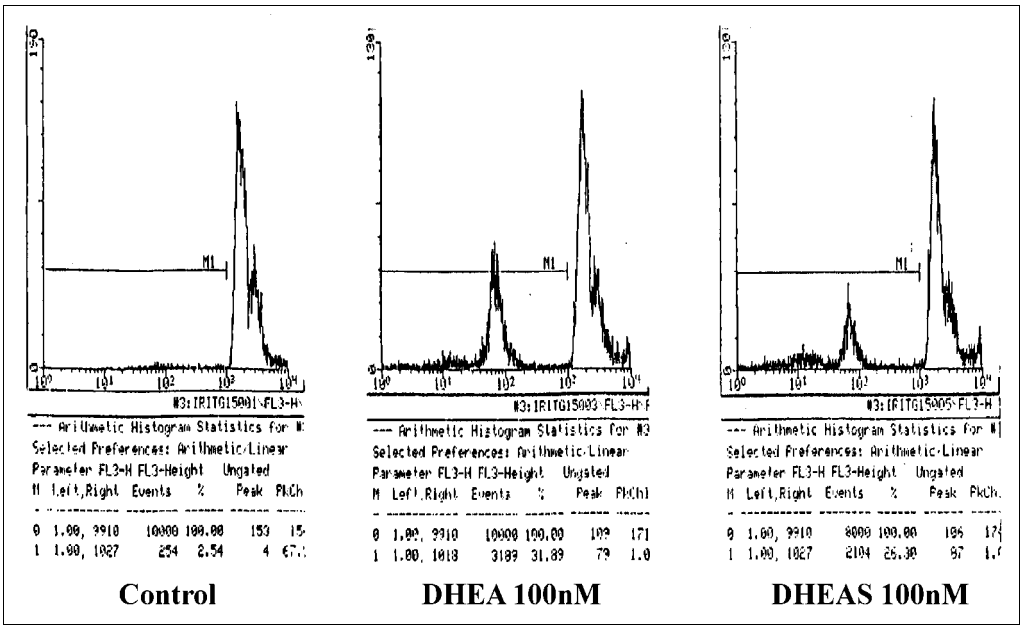

Figure 4. Flow cytometry profile of DNA in neuroblastoma cells stained with propidium iodide and treated with either DHEA or DHEAS (100 nM). Apoptosis rate was 2.5%, 31.9% and 26.3% in controls, DHEA and DHEAS groups, respectively.

another study, serum levels of DHEA/S did not correlate with health status, cognitive performance, or the presence of Alzheimer's disease in elderly subjects [18].

Our study demonstrates the differential effect of DHEA and DHEAS on the viability and proliferation of neuronal cells *in vitro*. While DHEA showed a neurotoxic and anti-proliferative effect on neuronal cultures, DHEAS did not alter or only slightly facilitated neuronal survival. DHEA alone, or in combination with DHEAS, did not have a toxic effect on whole-brain tissue. In contrast, previous reports have suggested a facilitatory role for the two neurosteroids in neuronal and glial cell survival, and a neuroprotective effect of DHEA against NMDA-induced neurotoxicity [5–7]. The variations in these studies and our results can be explained by the use of different experimental models. Our study showed an inhibitory effect of DHEA in both selected neuronal culture and neuroblastoma cell line. The maximal inhibitory effect was achieved at a low concentration of 10 nM–10 μM, but in whole-brain tissue it was ineffective. In the study of Bologa et al. [5] all experiments were performed on whole-brain culture. These authors also reported that higher concentrations of DHEA (1–100 μM) were ineffective in facilitating brain cell survival.

In the central nervous system, DHEA and DHEAS are present in the forms of non-conjugated steroids (free), sulfate ester and fatty acid ester. They are synthesized mostly in oligodendrocytes and affect central amino acid and neurotransmitter receptors [19]. Since the hormones are interconvertible in normal physiological conditions, DHEAS is present in the brain at much higher concentrations (10–100 nM) than DHEA. It can therefore be assumed that this homeostatic state prevents DHEA-induced neurotoxicity. The inability of DHEA to alter the viability of cells in whole-brain culture reflects the physiological status in the brain, and can be explained by the predominance of glial cells in culture. Astrocytes have been reported to contain high amounts of vitamin E, ascorbate and enzymes of the glutathione metabolism, which play a role in anti-oxidative processes in the brain [20]. In addition, glial cells secrete neurotrophic growth factors, such as glial cell line-derived, ciliary and nerve growth factor, which support neuronal survival by protecting neurons from different insults.

The possibility that a small portion of DHEA is converted in the primary whole-brain culture to DHEAS cannot be disregarded, since enzymatic activity of DHEA sulphotransferase was found in rat brain [21]. Such activity is also in line with the resistance to DHEA of whole-brain culture. Such activity, however, was not reported in selected neurons or in neuroblastoma cell lines.

The reported anti-tumor effect of DHEA is consistent with our findings of anti-proliferative and increased apoptotic rate in neuroblastoma cells exposed to DHEA, and to a lesser extent to DHEAS. At low concentrations, DHEA reportedly inhibited cell proliferation induced by estrogen in breast cancer cell lines [22] and arrested the cell cycle in colonic adenocarcinoma cell lines [23]. Some epidemiological studies have found a negative correlation between the plasma levels of DHEA and DHEAS and the risk of breast cancer in premenopausal women and of gastric cancer in patients at risk [24]. To explain the anti-mitotic activity of DHEA, Ebeling and Koivisto [9] hypothesized that its effect is exerted either as a weak estrogen or as an androgen, depending on the hormonal milieu of the tissue. Moreover, in a neuroblastoma cell line (SK-N-SH), beta-amyloid protein was reported to induce toxicity [25], while DHEA was found to stimulate the synthesis of serum amyloid precursor protein [17]. Finally, our results suggest a differential activity for DHEA and its sulfated analogue DHEAS – the first possessing a toxic and anti-proliferative effect and the second only a slight neuroprotective effect.

NMDA = N-methyl-D-aspartate

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