Dehydroepiandrosterone Inhibits Proliferation and Suppresses Migration of Human Cervical Cancer Cell Lines

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Abstract. *Dehydroepiandrosterone (DHEA), an adrenal steroid which is most abundant in human plasma, has a protective role against several types of cancer; however, its mechanisms of action are unknown. We evaluated the effect of DHEA on the proliferation and migration of three cell lines derived from cervical cancer. Cell proliferation was evaluated by crystal violet staining; migration by attachment, transwell and wound assays. DHEA inhibited the proliferation of InBl and SiHa cells, with a half-maximal inhibitory concentration (IC_{50}) of 30 μM, whereas the proliferation of HeLa cells was inhibited with an IC_{50} of 70 μM. DHEA at these IC_{50} inhibited attachment of cells to the plastic surface of the culture wells, and migration, was evaluated using transwells after 24 h of exposure. DHEA also reduced migration of the three cell lines into the wound area. These results suggest that a possible mechanism of DHEA in protecting against cervical cancer is the inhibition of proliferation and migration of tumor cells. DHEA could be useful in the prevention or treatment of cervical cancer.*

Cancer is currently an important public health problem in developing countries because it causes a high number of deaths annually. Cervical cancer used to be the leading cause of cancer death for women in the United States (1). In 2010 (the most recent year numbers are available), 11,818 women in the United States were diagnosed with cervical cancer therefore, it is very important to find new alternative therapeutic treatments.

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The main event leading to death in patients with cancer is cancer invasion and the development of metastases (2). Tumor metastasis is a multi-step process involving enhanced angiogenesis, altered cell adhesion, degradation of the extracellular matrix and cell migration, followed by intravasation of tumor cells to either the blood or lymphatic vessels, thus accessing the general circulation (3). After circulating in the vascular systems, tumor cells localize at distant organs where they establish secondary tumors or metastases. Invasion, which initiates the metastatic process, involves the participation of numerous biomolecules interacting in a variety of intricate cellular functions, including altered cell adhesion, proteolysis and migration (4).

Dehydroepiandrosterone (DHEA), an adrenal hormone, has been described to have a protective effect against cancer (5); however, the mechanisms involved with this effect have not been fully assessed. Recently, our group showed that DHEA inhibits the proliferation and migration of tumor cells derived from the breast (6). Other studies have shown that DHEA, at high concentrations (10-100 μM), inhibits *in vitro* endothelial cell proliferation, migration and capillary tube formation, and *in vivo* angiogenesis (7); its sulfate form (DHEA-S) inhibits proliferation and migration of vascular smooth muscle cells (8), as well as migration of human neutrophil and airway smooth muscle cells (9).

We hypothesized that DHEA inhibits proliferation and migration of other tumor cells such as cervical cancer. To test this, we evaluated the effect of DHEA on proliferation and migration of three cell lines derived from cervical cancer.

Materials and Methods

Materials. RPMI-1640, trypsin, TM and newborn calf serum (NBCS) were purchased from GIBCO/BRL (Franklin Lakes, NJ, USA). Sterile plastic material for tissue culture and transwells with inserts of 6.5 mm of diameter and 8 μm pore size, were purchased from Becton Dickinson, Immunocytometry Systems (San Jose, CA, USA). DHEA (trans-dehydroandrosterone, minimum 99%) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Cell culture. HeLa and SiHa cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), and InBl cells were obtained from a biopsy of cervical cancer tumor. SiHa cells are infected with HPV16, and HeLa and InBl cells with HPV18. All cells were grown in RPMI-1640 medium supplemented with 10% NBCS, L-glutamine (2 mM), and incubated at 37˚C in an environment of 5% CO_2-95% air.

Cell proliferation. Cell numbers were evaluated by crystal violet staining. Cells (5×10^3) were plated in 96-multiwell plates and cultured without and with different concentrations of DHEA (10, 20, 30, 40, 70, and 100 μM) or vehicle (1% ethanol) for 48 h. Afterwards, cells were fixed with 100 μl of ice-cold glutaraldehyde [1.1% in phosphate buffer solution (PBS) (150 mM NaCl, 4.4 mM KCl, 10.9 mM Hepes, 12.2 mM glucose, pH 7.4)] for 15 min at 4˚C. Plates were washed three times by submersion in de-ionized water, air-dried, and stained for 20 min with 100 μl of a 0.1% crystal violet solution (in 200 mM phosphoric acid buffer, pH 6). After aspiration of the crystal violet solution, plates were extensively washed with de-ionized water, and air-dried prior to the solubilization of the bound dye with 100 μl of a 10% acetic acid solution incubated for 30 min. The optical density of the plates was measured at 595 nm in a Quant multiplate microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Cell adhesion assay. This assay was carried out as described previously (10) with slight modifications. Cells were seeded in a six-well plate at a density of 2.5×10^5 cells/well in 2 ml of medium, and treated with DHEA for 24 h. After this, cells were detached with 0.025% trypsin, centrifuged, resuspended, and seeded again in supplemented medium at 5×10^4 cells/well. After 2 h of culture, non-adhered cells were eliminated by washing with PBS; adhered cells were evaluated by crystal violet staining.

Migration assays. Transwells: Cells were seeded into 8 μm pore-size transwell filters at 2.5×10^5 cells/well in 250 μl of NCBS-free medium. To the lower chamber, 1 ml of medium with 10% NCBS, without or with DHEA (100 μM) or vehicle was added. After 24 h of treatment, cells on the topside of the filter were removed by scrubbing twice with a tipped swab, and cells on the underside of the filter and cells adhered on the plate (migrating cells) were stained with crystal violet and counted.

Scratch motility assay (wound assay): To confirm the capacity of cells to migrate, cells were seeded and grown to confluence. Confluent cells were then grown in serum-free RPMI-1640 medium for 24 h. Before the assay, the medium was replaced with a serum-free medium overnight. After this, cell monolayers were scratched with a 200 μl-pipette tip to create a wound. Plates and cells were washed twice with serum-free medium to remove floating cells and then the cells were replenished with a medium containing 10% serum (control), DHEA, or vehicle. Cells migrating from the leading edge were photographed at 0 and 72 h. The percentage of open wound area (percentage of an image that is not considered occupied by cells) was calculated using TScratch software (11).

Statistical analysis. Data are presented as the average values±standard deviation of the mean (SD). Student's *t*-test was applied to determine statistical significance with acceptance at $p<0.01$.

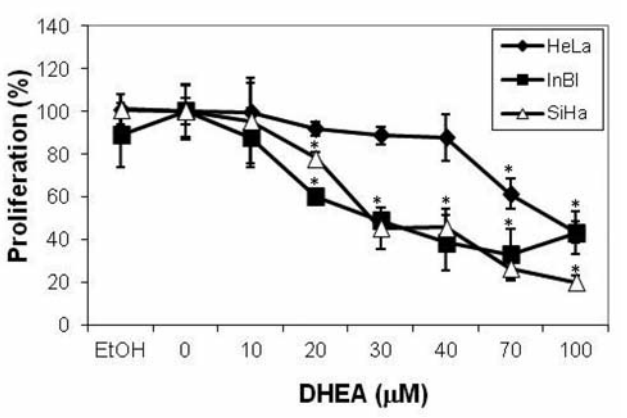


Figure 1. *DHEA reduced cell proliferation. Cells (5×10^3) were plated in each well and incubated with 10, 20, 30, 40, 70, and 100 μM DHEA for 48 h. Cell proliferation was assayed with violet crystal staining. Data are shown as the percentage of proliferation compared with the control (100%). The results are expressed as the mean±SD from three independent experiments, each performed with five replicates. EtOH: 1% ethanol was used as a vehicle for DHEA. *p<0.05 versus non-treatment control group.*

Results

DHEA reduced proliferation of cervical tumor cells. In order to determine if DHEA affects cell proliferation, cervical cells were exposed to different concentrations of DHEA for 24 h, and proliferation was evaluated by crystal violet staining. DHEA inhibited the proliferation of all cell lines in a concentration-dependent manner. Of the three cell lines, HeLa cells were the most resistant to the anti-proliferative effect induced by DHEA, with a half-maximal inhibitory concentration (IC_{50}) of 70 μM in comparison with an IC_{50} of 30 μM for InBl and SiHa cells (Figure 1). The IC_{50} obtained for each cell line was used in all the subsequent experiments.

DHEA reduced adhesion, migration, and motility of cervical cells. Migration and invasion of cancer cells through the basement membrane into the extracellular matrix are essential steps in cancer metastasis (12); therefore, we tested if DHEA could alter cellular adhesion. For this reason, cells were treated with DHEA for 24 h, detached and seeded again for 2 h, and cells adhered to the culture plate were evaluated by crystal violet assay. DHEA diminished adhesion of HeLa cells by around 30%. In InBl and SiHa cells, DHEA reduced adhesion by 50% and 35%, respectively (Figure 2). To test the ability of cells to migrate, transwell assays were performed. DHEA inhibited migration of InBl and SiHa cells by 50% and 25%, respectively; however, migration of HeLa cells was not significantly altered by DHEA (Figure 3). To test motility, cells were grown until forming an approximately

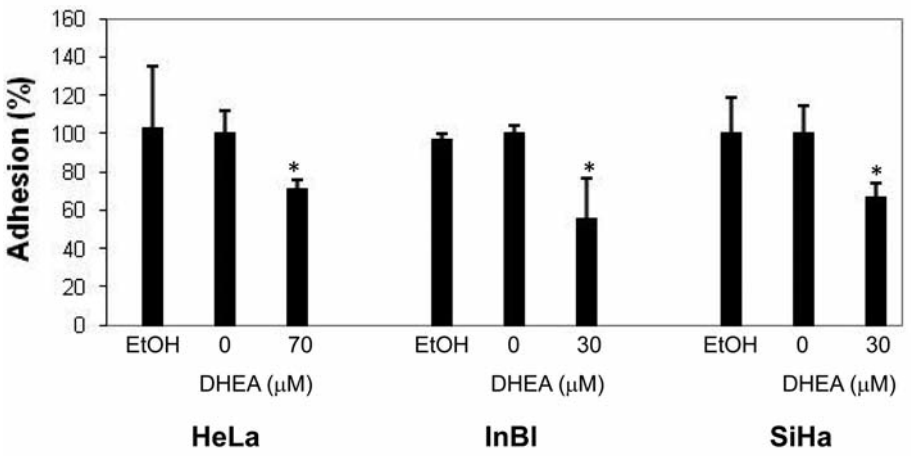


Figure 2. *DHEA reduced adhesion. Cells (2.5×10^5) were cultured with IC_{50} of DHEA for 24 h. After treatment, cells were detached with trypsin and re-plated again at a density of 50×10^3 cells/well for 2 h. At the end of incubation, non-attached cells were eliminated with two washes with PBS and adhered cells were stained with crystal violet, as described in the Materials and Methods section. The results are expressed as the mean±SD from three independent experiments. EtOH: 1% ethanol was used as a vehicle for DHEA. *p<0.05 versus non-treatment control group.*

100% confluent monolayer, and their capacity to move into a created space (wound) was evaluated. Wounds were completely closed after 72 h of culture with 10% NCBS (control). DHEA induced a significant reduction of around 50% in the width of the scratch in HeLa and InBl cells, and 70% in SiHa cells, as compared with control cells (Figure 4).

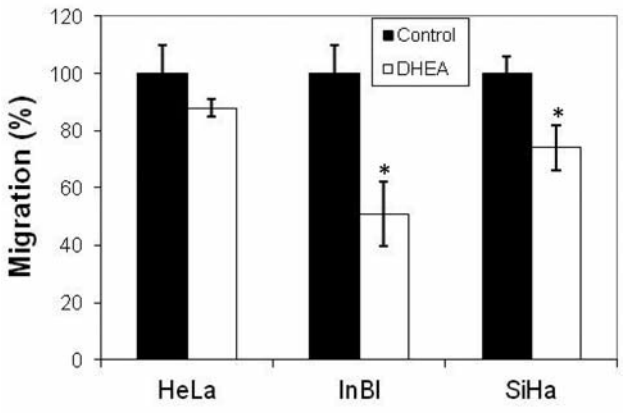
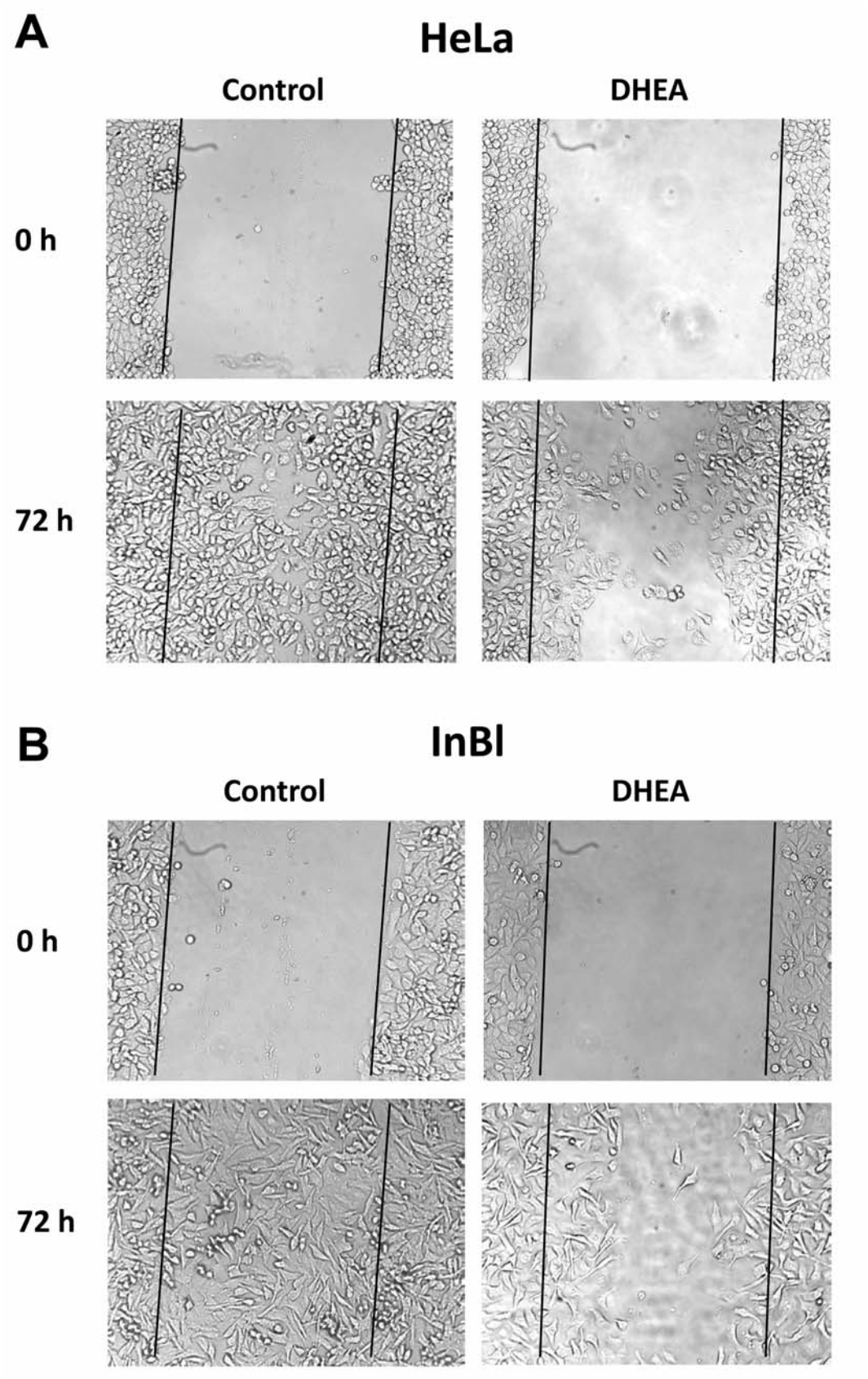


Figure 3. *DHEA reduced migration. Cells were cultured without (control) and with DHEA, and migration was evaluated by transwell assay at 24 h. Data are shown as the percentage of migration in comparison with control cells (100%), and are expressed as the mean±SD from three independent experiments. *p<0.05 compared to the control.*

Discussion

Migration of cancer cells from the tissue of origin to surrounding or distant organs is essential for tumor metastasis. Tumor cells invade the vasculature (a process termed intravasation), travel through the vascular system (blood or lymph) to a distant location and, in a reverse process, exit from the vasculature (extravasation) into an organ remote from the original site and establish a secondary tumor (13). Invasion and metastasis of cancer cells are the leading causes of mortality in patients with cancer; therefore, since a protective role of DHEA against cancer has been described, the effect of DHEA on the migration of cervical cancer cells was evaluated here.

Our results showed that DHEA inhibited proliferation, at pharmacological concentrations, of all three cell lines used. HeLa cells were the most resistant to the antiproliferative effect induced by DHEA; this might be related to the malignant state of the cells, because HeLa cells correspond to an advanced-stage cervical cell carcinoma (IV-B metastasis stage) (14), in comparison with InBl and SiHa cells, which represent stage IV-A and II, respectively. Many reports have shown that pharmacological doses of DHEA have chemopreventive and anti-proliferative effects on tumors in rodents (15). Our group showed that pharmacological concentrations of DHEA inhibit the proliferation of several tumor cells (16, 17), and of endothelial cells (18), indicating that DHEA inhibits proliferation of a wide cellular spectrum. We believe that the inhibition of proliferation is the first step to reduce adhesion and migration of tumor cells and prevent their metastasis. The anti-proliferative effect induced by DHEA on cervical cancer cells was associated with a decrease in cellular adhesion (attachment assay) and migration (transwell and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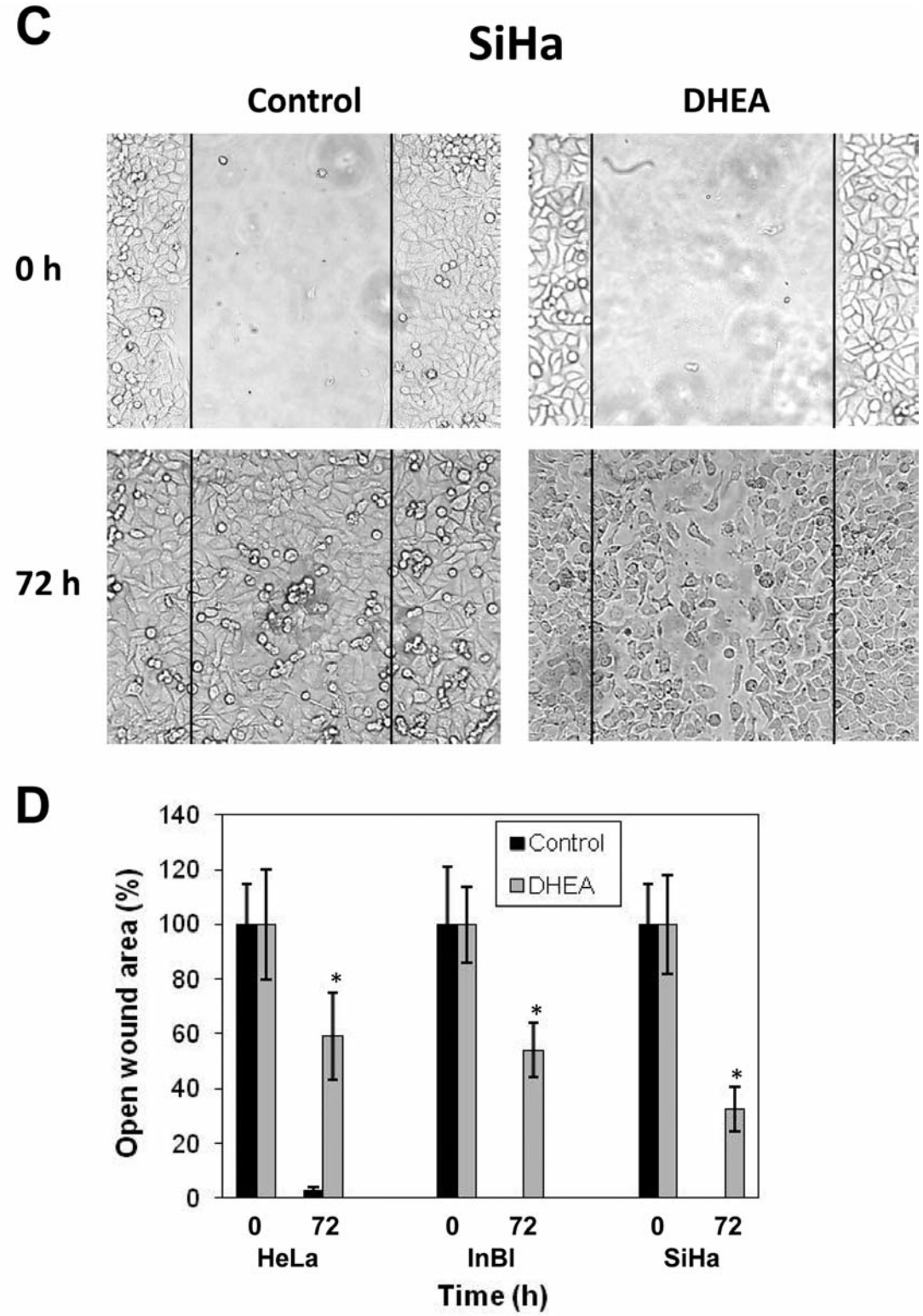

Figure 4. *DHEA reduced motility. Confluent monolayers from HeLa (A), InBl (B), and SiHa (C) cells were cultured without (control) and with DHEA, and cellular motility was evaluated by wound assay at 72 h. Photographs of cells were taken at 0 and 72 h after the treatments at ×10 magnification. D: An analysis of the percentage of open wound area was performed with TScratch software. Data are shown as the percentage of open wound area in comparison with control cells, and are expressed as the mean±SD from three independent experiments. *p<0.5 compared with the control at time=0.*

wound assays), which are mechanisms involved in the metastatic process in cancer. Similar results have been obtained by our group before, where DHEA inhibited the migration of breast tumor cells (6). However, there is only little experimental evidence about the effect of DHEA on cancer cell migration. In other studies, it has been shown that DHEA suppresses migration of eosinophils and their infiltration into the lung to improve the symptoms of asthma in ovalbumin-sensitized mice (19). On the other hand, the sulfate form of DHEA inhibits migration and proliferation of vascular smooth muscle cells (8, 20), indicating that DHEA has a wide capacity to inhibit migration of different cell types, and affect the metastatic process.

In conclusion, our results indicate that the inhibitory effect induced by DHEA on proliferation, adhesion, and migration could be related to the protective role of DHEA against cancer cells; therefore, DHEA might be of use alone or in combination with other chemotherapeutic drugs in the treatment of cervical cancer.

Acknowledgements

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