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Anti-aging effects of the proteins from artemia extract on human fibroblasts cell proliferation and collagen expression in induced aging conditions

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Abstract

Researches on the factors that inhibit senescence are attractive in health industries. Artemia extract uses in cosmetic and sunlight protecting products. The aim of this work was to study the effects of Artemia extract on the cell proliferation, senescence and collagen synthesis by human fibroblasts in normal and aging induced conditions. Artemia extract was extracted from napauliies and partially purified in 7 fractions. The human foreskin fibroblasts (normal and H_2O_2 stressed) were treated by crude and/or fractions in a dose dependent manner for 96 h. Cell counting, MTT and BrdU assays indicated that the fractions 4,6,7 induce cell proliferation. Treatment of cells by these fractions resulted in the reduction of senescence-associated ß-Galactosidase activity up to 50% in comparison to H_2O_2 treated control cells. The highest collagen type I mRNA expression was observed in fractions 5 and 7. The mRNA level increased in comparison with untreated and house keeping gene (β-actin) mRNA level. Percent of collagen type I expression to β-actin was 2.19 and 1.31 fold in cells which treated by fractions 5 and 7. Artemia extracts induce fibroblast proliferation, collagen synthesis and reduce cell senescence. These finding support the anti-aging effect of this product for probable application in the future.

Introduction

Aging and senescence are characterized by the reduction of response to stress, increasing homeostatic imbalance and increased risk of diseases [1]. Many factors such as genetic elements, free radicals, oxidants, Reactive Oxygen Species (ROS), telomeres shortening, alcohol and etc. influence and related to aging [2,3,4]. In replicative senescence, normal somatic cells invariably enter a state of irreversibly arrested growth and altered function after a finite number of divisions [5]. Evidences indicated that, this process is related to a tumor-suppressive mechanism and an underlying cause of aging [6].

In hemostasis some anti-aging factors, such as ascorbate, tocopherol and carotenoids neutralize aging inducing factors. These factors defenses against oxidative damages. Compounds from marine sources have been reported to have bioactive properties with varying degrees of actions such as anti-tumor, anti-cancer, anti-microtubule, anti-proliferative, anti-hypertensive, cytotoxic and antibiotic properties [7,8,9]. Researches on the new anti-aging factors that inhibit senescence are interesting option in cell biology, cosmetic production and health industries [10,11,12]. Artemia extract uses in cosmetic and sunlight pro-

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tecting products. Evidence indicated that the artemia extract contains tetra phosphate. Artemia uses it as energy source. P 26 protein from artemia possesses molecular-chaperone activity for RNA [13]. Artemia extract contains biologically active substances that cause increase in metabolism of skin cells and epidermal cell proliferation too. These evidences indicated that, artemia extract can provide anti-aging effects on cells and also harmonize with other anti-aging agents. Artemia found in a wide variety of hyper saline habitats ranging from desert to, tropic toand mountains. Artemia encysted and diapause exhibits a level of stress tolerance such as hyper salinity, very low oxygen tensions and extreme of temperature [14]. One of this species (Artemiaurmiana) lives in lake of Urmia in Iran. Lake of Urmia is one of the largest permanent hypersaline lakes in the world and resembles the great salt lake in the western USA in many respects of morphology, chemistry and sediments [15,16].

Mud of the Urmia lake and Artemia urmiana extract have been used in traditional medicine to treatment of skin inflammatory disorder The properties of Urmia Lake water and biomaterials derived from its organisms make it suitable for the treatment of various rheumatism diseases, skin disorders, metabolic disorders, women's diseases, and so on. The mud of this lake is black in color and have a boosting effect. The high concentration of salt in the lake also makes the floating on the water bring it relaxation to the individual. Skin collagen and joint diseases are also treatable with artemia extract, black mud and salt. Among other therapeutic properties of Urmia lake living organisms, treatment of respiratory diseases such as asthma and bronchitis and various sinusitis can also be mentioned. Urmia Lake water is one of the watersheds of Sodium chloride. Drinking this water reduces bile concentrations and as a result, bile secretion improves better [17]. The extract uses in cosmetic and health industries. Literature review indicates that the content and mechanism of action of its active materials poorly studied. Therefore the aim of this work was to evaluate the anti-aging effect of artemia extract in cellular level *in vitro*. For these purposes; we isolate and purified the proteins of *A. urmiana* extract. The effects of partially purified proteins on the cell growth, senescence and collagen type I expression of human foreskin fibroblasts were studied.

Materials and methods

All chemicals which used in this study were provided from Merck (Merck, Darmastd, Germany) except which indicated from other sources otherwise and separately.

Cyst hatching

A*. urmiana* cysts were provided from Urmia lake (west-north of Iran). 10 gr of cysts were hydrated in water at room temperature for one hour. Then the cysts were decapsulated by Sodium Hypocholorate (NaOCl 2.5%) for 2-3 min to change the colure of cysts from brown to orange. Then the cysts were washed by 500 ml of cold distilled water. The encapsulated cysts were hatched in 2 liters of artificial sea water (0.4 M NaCL , 0.009 M KCL, 0.05 M MgCL2, 0.009M $CaClL_2$ and 0.028 M Na_2SO_4, pH=8.0) according the method reported by Rottini [18]. The cysts were incubated at 28ºC for 24 hr under light chamber in a shaking incubator. Freshly hatched napulii are phototropism, therefore they were collected by attraction to light). The collected napuliies were washed with 400 ml of cold distilled water. Embryos were suspended in 50 ml of stock solution of A [19] pH=7.6 and sonicated three times for 30 second. Then stock solution of B (1/9) was added and centrifuged at 20000g for 20 minutes (Heraeus, Hanau, Germany). The supernatants were filtered through two layers of Mira cloth. The filtered homogenate was keep at -20ºC for protein purification. In all of experiments total protein concentration was measured by modified Bradford methods [20]. The proteins content of crude extracts was precipitated with salt extraction by using Ammonium Sulfate 40% (W/V) at 4°C by shaking for 2 hr. The precipitate was centrifuged at 5000 rpm for 20 minutes (Heraeus, Hanau, Germany) for 20 min at 4°C. Then the pellet was dissolved in dialysis buffer (Tris-HCl 50 mM, pH 8.7) and dialyzed against the same buffer for 24 hrsat 4 ºC by 2 times change of the buffer.

DEAE-Sepharose anion-exchange chromatography

A 18 x 3 cm DEAE Sepharosecolumn (What man, Maidste, England) was used for chromatography. First, the column was extensively washed by binding Tris buffer (Tris-Base 50 mM, pH 7.5) for equilibration. Subsequently, protein was eluted applying elution buffer (100 ml of Tris-Base 50 mM, pH 7.5). Then salt gradient was applied by adding 400ml of 0.1 to 1.0 M of NaCl in Tris-Base 50 mM buffer pH 7.5by flow rate about 1 ml/min. Each 3.0 ml of samples was collected and monitored at 280 nm continuously. The samples of each fraction were collected together and dialyzed. The final total protein concentration was measured by modified Bradford methods. Finally, the fractions were concentrated and sterilized by 0.2 µm filter papers (What man, Mailste, England) to use for further analysis in cell culture system.

For analysis of the proteins profile, crude extract and different fractions of chromatography were electrophoresedon 13% resolving SDS-PAGE gel based on the buffers used by Laemmeli [21]. The electrophoresis was done by LKB-Pharmacia electrophoresis system (LKB Pharmacia, Uppsala, Sweden). After running; the gels were stained by coomassie blue.

Human foreskin fibroblast isolation and culture

Primary human fibroblasts cells were isolated from human neonatal foreskins by inform and consenting donors. As the donors were neonates, written, informed consent was obtained from the legally authorized parents of the donor neonates for using foreskin surgery tissuesat the Mustafa Khomeini hospital prior to the study. In order to isolate fibroblasts, the other intruding peripheral tissues were removed. The tissue was chopped in 2-3 mm dissected into small sections. These small fragments were incubated in the collagenase solution (1.1 U/ml) (Gibco, Paisley, United Kingdom) at 37°C for 50 min. The mixture was centrifuged (300×g for 5 min at 4 °C). The cells were cultured in dulbecco's modified eagle's medium (DMEM) supplemented by 10% fetal bovine serum (FBS) (Gibco, Paisley, United Kingdom). Cultures were maintained at 37 °C with a humidified atmosphere of 5% CO_2. Culture media were changed twice a week until the culture density reached to 80 % confluency. Then, the cells were trypsinized and passaged regularly. In all experiments, cells in passages 4 to 6 were used for treatment study.

Oxidative stress induction of cells by H_2O_2 and later treatment by artemia extracts

Primary human fibroblasts (3×10^4cells/cm^2in DMEM) were separately treated by H_2O_2 (6.5 µM) for 2 hr. Then the cells were washed and treated by increasing concentration of artemia extract (0,5,25,50,100 µg/ml) in 5 mL of DMEM medium containing 10 % of FBS in 6-wells plates (Nunc, Roskilde, Denmark). The cells were incubated at 37°C for 72 h. Then the cells and

supernatant of the treated cells were collected separately. The effect of artemia extracts on the cell growth, cell proliferation, cell senescence and expression of human Collagen type Iwere done as describe below.

BrdU and MTT cell proliferation assay

In order to examine the effect of artemia extract on the cell growth and cell proliferation, the cells were collected after 72 h of treatment and the total cell number, cell viability, Bromodeoxyuridine (BrdU) and MTT cell proliferation assays were done. The cell number and viability were enumerated using a Neubauer hemocytometer. BrdU cell proliferation assay was done using the BrdU cell proliferation ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly 3×10^3cells (in100µl/well) of control and treated cells were transferred to 96-well micro titer plate**.** 10 µl of BrdU solution was added to each well and incubated over night. Then the micro wells were centrifuged and dried by hair drier**.** 200 µl of Fix Denat was added to each well and incubated for 30 min at 15–25°C. After removing of FixDenat, 100µl/well of anti-BrdU-peroxidase conjugated antibodies were added and incubated for 90 min at room temperature. The solution was completely removed and the wells were washed three times with 200 µl of washing buffer. Finally, 100µl of tetramethylbenzidine (TMB) substrate solution was added and incubated at room temperature for 15 min and reaction was stopped by adding 100µl of stop solution. The absorbance were measured at 550nm by ELISA reader (Labsystems Multiskan, Roden, Netherlands). In MTT assay, 3×10^3 untreated and treated cells (in 100µl/well) were transferred to flat bottom 96 micro titer plates. Then 10 µl of freshly prepared [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) (Sigma, St. Louis, MO, USA) solution (5 mg/ml in PBS) was added to each well and were incubated for 4 h. Finally, supernatant removed then 50 µl of DMSO solution was added to each well. Absorbance was read at 580 nm using an ELISA reader (Labsystems Multiskan, Roden, Netherlands).

Senescence sensitive β-galactosidase staining

Cytochemical staining for acidic senescence associated β-galactosidase (SA- β-gal) was performed as described by Gary and Kindell with some modification [22]. Briefly treated cells by H_2O_2 and/or artemia extract were collected and washed by PBS. Then the cells were fixed for 3-5 min in a fixative (2% formaldehyde, 0.2 % glutaraldehyde in PBS). The the cells were incubated for 12 hr at 37°C with freshly prepared β-galactosidase staining solution: 1 mg/ml of 5-romo-4-Chloro-3-Indolyl- β-D-Galactopyranose (X-Gal) (Sigma, St. Louis, MO, USA), 20mg/ml of dimethylformamide, 40 mM citric acid/sodium phosphate, pH 6.0, 5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanade,150 Mm NaCl, 2 mM $MgCl_2$. Finally, the substrate was collected and the cells were washed. The absolute of blue stained cells was observed directly by eye microscopy and percent of stained cells were counted in minimum of 300 cells.

Semi-quantitative detection of collagen type I by RT-PCR

Artemia extract treated fibroblasts were collected and total RNA was extracted by using the tripure isolation reagent (Roche, Mannheim, Germany), according to the manufactures protocol. RNA yield and purity were quantities by measuring optical density ($OD_{260/280}$) using a spectrophotometer Beck man DU530 (Beckman Coulter Inc. CA, USA). First-strand cDNA was synthesized from 1 µg of total RNA using Maurine Maloney Leukemia virus (M-MLV, Fermentase) and reverse transcriptase (Fermentase Gmbh, Leon-Rot, Germany) with oligo-dT primer (Fermentase Gmbh, Leon-Rot , Germany), according to the manufacturer's instructions. The collagen type I cDNA and β-actin cDNA were amplified by the following primers:

Collagen type I forward primer:

5'CCCCCTCCCCAGCCACAAAG-3'

Collagen type I reverse primer:

5'-TCTTGGTCGGTGGGTGACTCT-3'

β-actin forward: 5'-AAGAGAGGCATCCTCACCCT-3'

β-actin reverse :5'-TACATGGCTGGGGTGTTGAA-3'.

The expected sizes of the PCR product were 360bp for collagen and 220 bp for β-actin. The thermal cycling conditions for amplification of collagen and β-actin fragments were as follows: 94°C for 5 min., followed by 35 cycles at 95°C for 60s; 56 °C for 60s; 72°C for 90s. This was followed by re-extension at 72°C for 10 min. The PCR products were separated on a 1.8% agarose gel (using 0.5 × TBE buffer) and visualized by ethidium bromide staining.

Statistical analysis

Each experiment was minimally performed three times for all data, each carried out in duplicated sequences. Data were analyzed using a One-Way Analysis of variance (ANOVA) Values were given as the mean ± Standard Deviation (SD) and analytical variables were compared by using the students' t-test. By convention, a α-level of $p<0.05$ was considered to be statistically significant

Results

Protein extraction and purification

Freshly isolated napuliies were collected, and suspended (0.2 gr/ml) in extraction buffer. The amounts of extracted proteins were about 17.6, 40.6 and 16 (24.7 ± 13.7) mg/gr of napuliies wet weight respectively. SDS-PAGE electrophoresis was done for analyses the protein profile of crude extract (Figure 1).

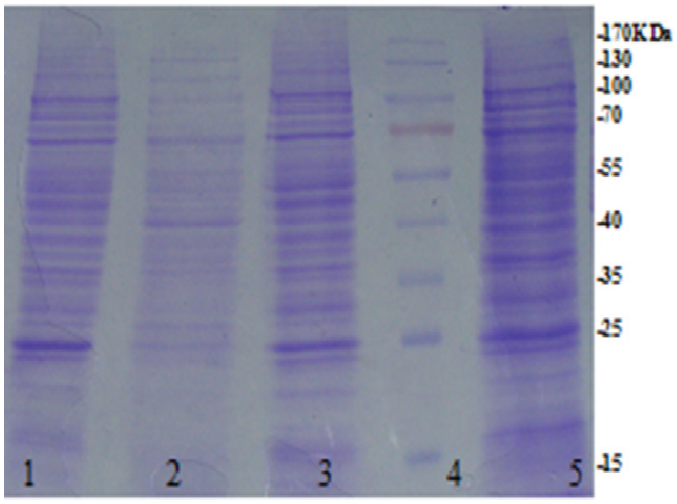
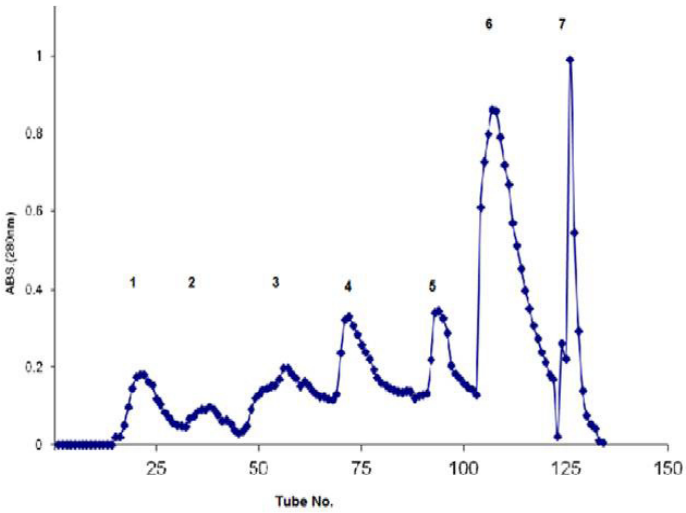


Figure 1: The SDS PAGE of A. urmiana extract. Crude extract was electrophoresed on 13% resolving SDS-PAGE gel. Line 1,3,5 indicated protein pattern of napaullies after 18 h of hatching. Line 2 indicated protein pattern of napaullies after 24 h of hatching. Line 4 protein MW marker.

Partial purification was done by DEAE sepharose ion exchange chromatography as described in methods. The samples were collected andmonitored at 280 nm continuously. Figure 2a. indicated to the chromatogram of the purification. Seven obvious fractions (1-7) were observed in the chromatogram. The fractions 1 and 2 belong to the cationic proteins which didn't bind to DEAE resin. Fractions 3, 4, 5, 6 and 7 resulted to the salt gradient elution of 0.1, 0.2, 0.3, 0.4, 0.5 and 1 M of NaCl. Different fractions of chromatography were electrophoresed on 13% resolving SDS-PAGE gel (Figure 2b). The purified fractions were filtered by 0.22µm, concentrated by freeze-drying and used for treatment and studying of anti-aging effects on fibroblasts.



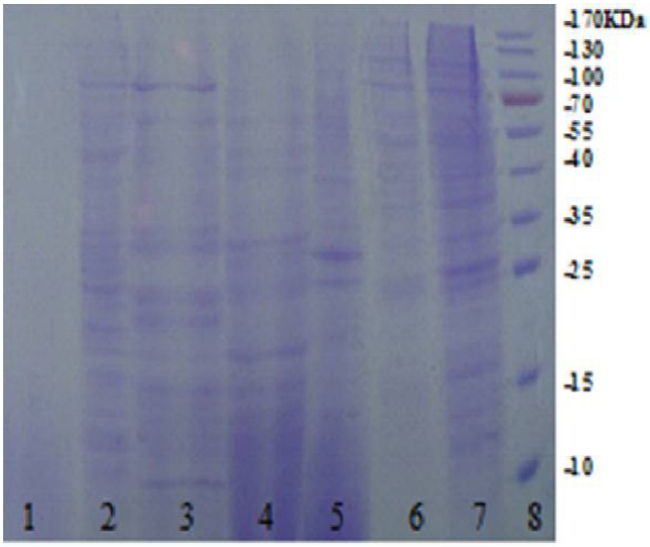


Figure 2: The chromatogram of DEAE anion exchange chromatography of Artemia extract and SDS-PAGE electrophoresis of the purified fractions. a) Chromatogram of the chromatography; fractions 1 and 2 belong to the cationic proteins, fractions 3, 4, 5, 6 and 7 resulted to the salt gradient elution of 0.1, 0.2, 0.3, 0.4,0.5 and 1 M of NaCl. b) Different fractions of chromatography were electrophoresed on 13% resolving SDS-PAGE gel. Line1-7 are samples from fractions 1-7 respectively, line8is prestained protein ladder SM0671 fermentase

Cell proliferation

The effects of artemia extracts (crude extract and 7 fractions after chromatography) on cell proliferation in normal and stressed condition were assayed as described in methods.The values for each testwere averaged and growth curves were constructed. Results indicated that fractions 3,5 and 6 increased cell proliferation in comparison to untreated cells and artemia crude extract treated cells by MTT assay (Figure 3a). Fractions 4, 6 and 7 showed increase in BrdU incorporation and entrance of cells to S phase of cell cycle in comparison to cells treated by crude extract and control cells (Figure 3b). Results indicated to increasing of cell growth potential and cell proliferation parameters up to 40% in the presence of artemia extract fractions (3,4,5,6,7) in comparison with untreated control cells.

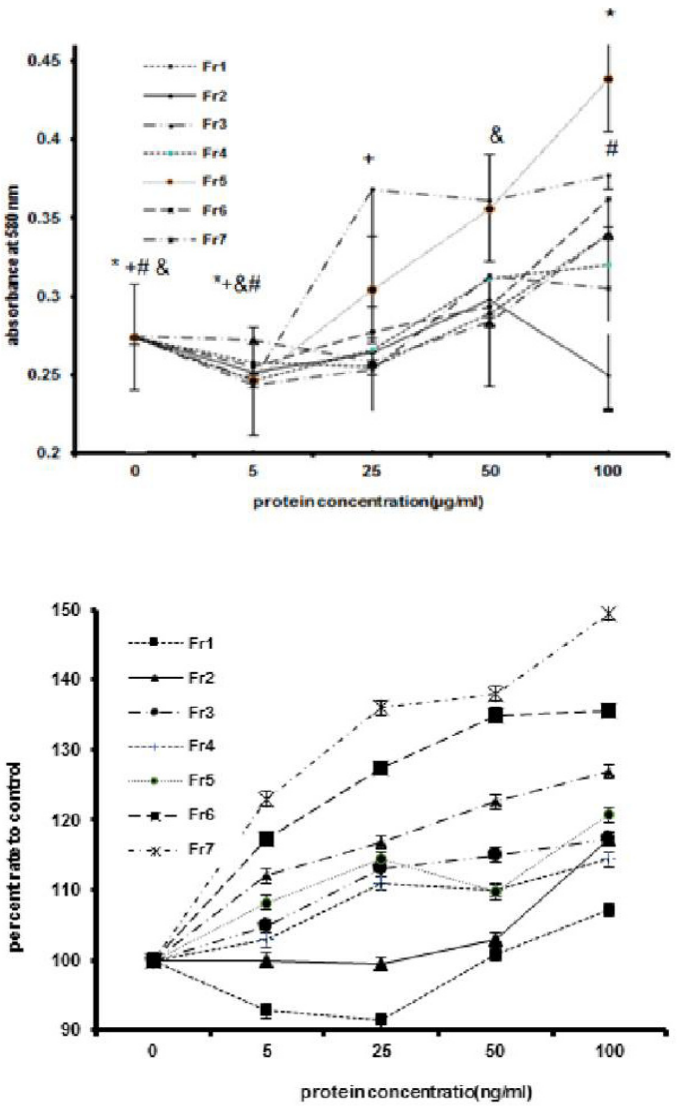


Figure 3: The effect of Artemia extract and purified fractions on the cell growth and cell proliferation of human fibroblasts by BrdU and MTT assay. The cells were grown in DMEM medium in the absence or presence of different concentrations of artemia extract purified proteins for 72 h as described in "Materials and Methods". At the time point, control and treated cells were collected: a) MTT assay of artemia extract and partially purified fractions. b) BrdU cell proliferation assay. The results are mean ± S.E.M. for three separate experiments.**+, &, *** indicated to the significance of the concentrations of 25, 50 and 100 µg/ml of fraction 7 in comparison to untreated control cells and cells treated by 5µg/ml of fraction 7 ($p<0.01$). **#** indicate to significance of the concentrations of 100 µg/ml of fraction 6 in comparison to untreated control cells and cells treated by 5µg/ml of fraction 6 ($p<0.01$).

Senescence sensitive β-Galactosidase staining

The cells (normal and 2 hr H_2O_2 stressed conditioned fibroblasts) were incubated with Artemia extract for 72 hr and assayed for β-Galactosidase activity as described in methods. Light microscopy confirmed the slightly inhibition of senescence with typical features of senescence. These features include an increased cell size and cytoplasmic granularity in blue stained senescence cells in comparison to untreated cells (Figure 4a). The stained cells were counted and the percent of stained cells were calculated (Figure 4b). The results showed that the addition of artemia extract (fractions 5, 6 and 7) reduced the number of senescence β-Galactosidase positive cells up to 50% in comparison to H_2O_2 treated control cells.

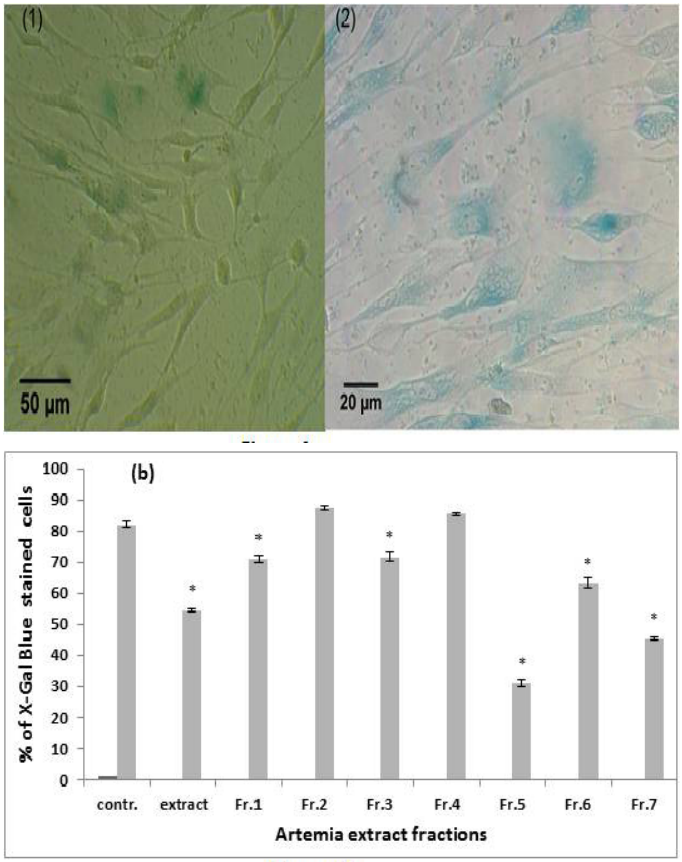


Figure 4: Cytochemical staining for acidic senescence associated β-Galactosidase (SA- β-gal. Treated cells by H_2O_2 and/or Artemia extract were collected and stained by X-Gal as described in "Materials and Methods". The absolute of blue stained cells was observed directly by light microscopy and percent of stained cells were counted in minimum of 300 cells. a) Light microscopy figures of β-Galactosidase staining blue stained cells 1) low senescence cells (250 X) 2) high senescence cells. (400 X) b) Percent of β-Galactosidase positive cells. The results are mean ± S.E.M. for three separate experiments. * indicated significance of results in comparison to crude extract p-values <0.01.

Expression of collagen type I

For analysis of collagen type I mRNA expression by fibroblast in response to artemia extract RT-PCR was performed by using RNA from untreated and treated cells (in stress condition with H_2O_2 6.5 µM). The quantity of the target was normalized by an endogenous reference (β-actin) relative to the calibrator (untreated cells). This method revealed the expression of collagen type I mRNA, as defined by a 360bp PCR product in the fibroblasts. Control cells (untreated cells) showed weak expression of collagen (Figure 5a). The results showed that artemia extract treatment increased collagen synthesis in compared with untreated control cells. The highest collagen mRNA expression was observed in cells which treated by fractions 1,5,6 and 7. The collagen mRNA level was quantified by house/keeping gene as described in methods. The results were shown in figure 5b. The results indicateto increase of collagen expression in cells which treated by fractions 1,5,6 and 7in comparison with untreated and house/keeping gene (β-actin) mRNA level. As shown the percent of collagen type I expression to β-actin was 0.3, 2.19, 0.42, and 1.31 fold in cells which treated by fractions 1,5,6 and 7.

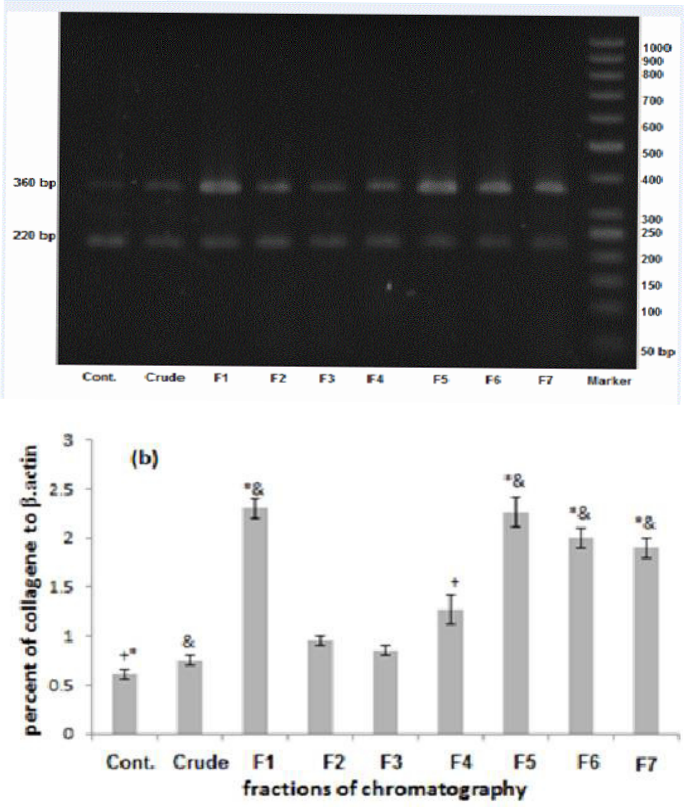


Figure 5: Changes in collagen type I gene expression of human fibroblast after treatment by artemia extract. The cells were stressed by H_2O_2 for 2 h and then incubated in presence and absence of artemia extract for 72 h as described in "Materials and Methods". Total RNA was extracted from untreated and treated cells and analyzed by RT-PCR for collagen type I gene expression. β-Actin was served as an internal house keeping gene control. a) Gel electrophoresis of RT-PCR products from untreated and treated cells by crude extract and fractions 1-7. DNA MW marker by 50 and 100 bp. 4b) Calculated percent of RT-PCR products of the collagen type I expression to β-actin of the above gel electrophoresis.&, * indicated to the significance of the fractions 1,6 and 7 in comparison to untreated control cells and cells treated by crude extract ($p<0.01$). + indicated to the significance of the Fraction 4 in comparison to untreated control cells ($p<0.05$).

Discussion

This study was focused on attempting to understand the effect of the artemia extract in stress condition on the cell proliferation of fibroblast cells. We have treated the fibroblast cells with artemia extract in stress condition for 72 hr. A significant behavior was observed on the cell proliferation after treatment of cells by fractions 3,4,5,6,7.

Fibroblasts (the most common cells of connective tissue) are type of cells that synthesizes the extracellular matrix contents specially collagens, as the structural framework (stroma) in ani-

mal tissues. They plays a critical role in wound healing. Collagen is the main structural protein of the various connective tissues in animals. Changes in protein structure during aging altered the morphology and structure of the skin as wrinkles, loss of elasticity and stiffness. The researches in the field of aging and anti-aging indicated that, stress with H2O2 have a variety of effects on growth and other cellular and biochemical characteristics of normal human skin fibroblasts. H2O2in mild stress induces oxidative damage of DNA, lipids peroxidation, Reactive Oxygen Species (ROS) levels and antioxidant enzymes activity in orbital fibroblasts. The response of cells to this mild stress is the activation of one or more Stress Response (SR) pathways and stimulation of repair mechanisms consequently [6]. Our result indicated that some of artemia extract fractions can neutralize the oxidative damage of H_2O_2 in fibroblast by stimulation of collagen type I expression. In this research we found that artemia extract and its, partially purified protein fractions have anti-aging effects as stimulation of the cell proliferation by fractions 3,4,5,6,7 and anti-senescence effects offraction 5,7. These effects accompanied by stimulation of the collage type I expression in treated cells by fractions 1,5,6,7.

Skin care cosmetic is one of the first successful products development which contain artemia extract as ingredient. Our results showed that the artemia extract had anti-aging and renewing effects on collagen. We can probably suppose that the existence and use of it in cosmetic product have anti-aging factor beside energy sources. Artemia extract induces Hsp70 in human cells and enhances cell protection from stress. Rattan et al. have reviewed a new multifunctional and compatible anti-aging cosmetic ingredient from marine extract. The ingredient is obtained from zooplankton, specifically the species Artemia salina. The main component of artemia extract is diguanosine tetraphosphate (GppppG) [11]. This is known as the source of energy for Artemia salina to develop the protective capacity against environmental stress. As a cosmetic ingredient, this extract can probably boost the generation of the Extra Cellular Matrix (ECM) proteins and cyclic 3', 5'-adenosine monophosphate (cAMP). In effect, artemia extract boosts the effects of other anti-aging agents.

Conclusions

Mud of the Urmia lake and Artmia extract from there have been used in traditional treatment of inflammatory skin disorder. From the last decade to now it uses in cosmetic and health industries. The mechanism of action was not clearly understood. Our in vitro result by partially purified artemia extract proteins showed that some of fractions can induce cell proliferation of human skin fibroblasts, inhibit cell senecense and stimulate collagen type I expression.

Acknowledgements

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Ethical approval

The study protocol was approved by the Research Ethics Committee at National institute of Genetic Engineering and Biotechnology (Tehran-Iran).

Informed consent

Primary human fibroblasts cells were isolated from human neonatal foreskins by inform and consenting donors. As the donors were neonates, written, informed consent was obtained from the legally authorized parents of the donor neonates for using foreskin surgery tissues prior to the study.

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