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The Influence of Iron Loading and Iron Chelation on the Proliferation and Telomerase Activity of Human Peripheral Blood Mononuclear Cells

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ABSTRACT

Background: Iron is an essential trace element in cell proliferation. Several investigations demonstrate that iron deprivation inhibits cell proliferation. However, the impact of iron on telomerase activity of activated lymphocytes remains unexplained to date. **Objective:** In this study, the effect of iron on the proliferation and telomerase activity of lymphocytes stimulated by phytohemagglutinin (PHA) were investigated. **Methods:** Iron loading was performed by incubating peripheral blood mononuclear cells in 500µM $FeSO_4.7H_2O$ for 24 h and iron chelation was done by exposing cells to desferrioxamine, a potent iron chelator. The effects of silymarin, a flavonoid with both antioxidant and iron chelating activities, on the proliferation and telomerase activity of PHA-activated lymphocytes were also compared with desferrioxamine. Proliferation and telomerase activity were assessed using BrdU incorporation assay and Telomeric Repeat Amplification Protocol (TRAP), respectively. **Results**: The proliferations of lymphocytes were significantly inhibited by 10 and 20 µg/ml desferrioxamine in a dose dependent manner, while iron loading recovered suppressed cell proliferation to the normal level. Silymarin at 20 µg/ml significantly increased the proliferation of lymphocytes in both normal and iron-treated conditions. Telomerase activity of lymphocytes was markedly increased by iron treatment and suppressed by desferrioxamine. Conversely, iron treatment had no effect on the telomerase activity of lymphocytes incubated with silymarin. **Conclusion:** Iron plays a significant role in the proliferation and telomerase activity of lymphocytes. The effects of silymarin on the proliferation and telomerase activity of lymphocytes were completely different from those of desferrioxamine, suggesting that the immunomodulatory effect of silymarin is probably not associated with its iron chelating activity.

Keywords: Telomerase, Iron, Desferrioxamine, Silymarin, Lymphocytes

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INTRODUCTION

Iron is the most abundant transition metal in living organisms. Cells of nearly all forms of life require a known amount of iron for survival, replication and expression of differentiated processes. Since, iron is an essential growth factor and a rate limiting trace element in DNA synthesis, iron homeostasis is crucial to normal cell metabolism, and its deficiency or excess is associated with numerous disease states (1,2). Desferrioxamine, a siderophore originally isolated from *Streptomyces pilosus*, is the chelator currently used to treat iron overload disease. Besides having anti-proliferative activity against tumor cells, desferrioxamine is a potent in vitro inhibitor of DNA synthesis by human B and T lymphocytes, preventing cells from completing the S phase of the cell proliferation cycle (3,4).

The telomere is a DNA protein complex that is essential for the stabilization of chromosomes. Telomerase is a specialized reverse transcriptase that functions to extend telomere sequences at the ends of linear chromosomes. In the absence of telomerase, telomeres shorten with each cell division. Human somatic cells, including T lymphocytes, have a limited lifespan and stop proliferating after a fixed number of divisions. In order to maintain proliferative capacity and avoid replicative senescence, lymphocytes are capable of upregulating telomerase upon activation, and possibly protecting proliferating lymphocytes of telomere shortening (5,6). Along with cell division, oxidative damage may affect telomere length through modulation of telomerase activity. Telomeres are highly sensitive to oxidative stress, which produces DNA single strand breaks. Several investigators indicate that telomere attrition is modulated by oxidant–antioxidant balance. Data from in vitro studies indicate that the guanine-rich telomeric repeats are the preferred sites for sequestration of transition metals that may participate in the generation of the oxidizing species responsible for strand breaks (7,8).

Flavonoids, which are phytochemicals produced by various plants in nature, have been revealed to protect biological membranes against free radical induced oxidative damage. Silymarin, a flavonolignan complex isolated from milk thistle (Silybum marianum L. Gaertn), is mainly composed of a mixture of silybin, silychristin and silydianin. These compounds are used mostly as hepatoprotectants and were shown to have anticancer, antioxidant, and anti-inflammatory activities (9). Moreover, a recent study has demonstrated that silybin, the silymarin component with the greatest degree of biological activity, acts as an iron chelator (10). Indeed, the polyphenol structure of silybin allows both the scavenging of free radicals and the chelation of transition metals, including iron. The question therefore arises as to whether the biological activities of silymarin are primarily due to their radical scavenging or iron chelating properties.

Since iron is a rate limiting trace element in DNA synthesis and is known to be a catalyst in the formation of reactive oxygen species (11), the present study was undertaken to compare the immunomodulatory effects of silymarin, a flavonoid with both antioxidant and iron chelating activities, with desferrioxamine, a potent iron chelator. To explore whether the biological activity of silymarin and desferrioxamine were consistent with an iron-depleting mode of action, the effects of silymarin and desferrioxamine on the proliferation and telomerase activity of iron-treated lymphocytes were investigated.

MATERIALS AND METHODS

Preparation of Human Peripheral Blood Mononuclear Cells (PBMC). Blood samples were collected from the blood of healthy volunteers in heparinized tubes and PBMC

were isolated using standard Ficoll-hypaque density centrifugation. The protocol for the present study was approved by the Ethics Committee of the Isfahan University of Medical Sciences. The interface lymphocytes were washed twice with phosphate-buffered saline (PBS). Cell viability was assayed by trypan blue exclusion (0.4% trypan blue in PBS) and cells with viability ≥98% were used in the test.

Cell Culture and Treatments. PBMC were maintained in completed media containing RPMI- 1640 (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (Gibco-BRL, Germany), penicillin (100 U/ml) and streptomycin (100 µg/ml) under standard culture conditions (37°C, 95% humidified air and 5% CO_2).Silymarin (Sigma St. Louis, USA) was dissolved in 100% dimethyl sulfoxide (DMSO) to 1 mg/ml concentration. The stock solutions were aliquoted and kept at −20°C for a maximum of 30 days. All subsequent dilutions were made in the RPMI medium. A stock of 1 mg/ml desferrioxamine (Sigma St. Louis, USA) was prepared in deionized water and sterilized using a 0.2 µm Millipore filter. Desferrioxamine stock solution was kept at 4 °C, and various dilutions were made in RPMI medium during the experiments. Lymphocytes were activated by 10 µg/ml phytohemagglutinin (PHA) (Sigma, USA) and treated with silymarin or desferrioxamine at 10 or 20 µg/ml for 3 days. Negative control cells were treated with DMSO and RPMI medium. The final concentration of DMSO in control wells was equal to those of the experimental samples.

Iron Loading of PBMC. Iron loading was performed as described by Traore and Meyer (12). Briefly, 1 ml of 1×10^6 cells/well were seeded in a 24-well plate in the presence of 500 µM FeSO4.7H2O and incubated for 24 h. The controls contained cells and media alone. Following iron incubation, cells were washed twice with PBS to remove nonabsorbed material and used for further experiments.

Lymphocyte Proliferation Assay. The proliferation of PHA-activated Lymphocytes was assessed by bromodeoxyuridine (BrdU) incorporation using a colorimetric ELISA kit (Roche, Germany). Cells (1×10^5) were cultured in 96-well plates in a final volume of 200 µl for 3 days. Subsequent to labeling with 10 µM of BrdU, DNA was denatured and cells were incubated with anti-BrdU monoclonal antibody prior to the addition of substrate. The reaction product was quantified by measuring absorbance at 450 nm.

PCR-ELISA Telomerase Assay. Quantitative determination of telomerase activity in lymphocytes was performed using the Telomeric Repeat Amplification Protocol (TRAP). For this purpose the Telomerase PCR ELISA kit (Roche Diagnostic GmbH Mannheim, Germany) was used. Telomerase activity present in lymphocyte extracts was detected following manufacturer's instructions. Briefly extracts were obtained from 2×10^5 cells using 200 µl of lysis buffer and centrifuged at 16000 ×g at 4°C for 20 min. The supernatant of cellular extract was used to evaluate the telomerase mediated addition of telomeric sequence. The products obtained were amplified by PCR. The amount of hexamers in each sample added by the telomerase was then evaluated by ELISA technique using TTAGGG specific probes. The reaction product was quantified by measuring absorbance at 450 nm (reference wavelength 690 nm). Immortalized telomerase-expressing human kidney cells (293 cells, provided by the supplier) were used as positive controls, and heat-treatment of the cell extract for 10 min at 65°C prior to PCR was used to inactivate telomerase protein to be used as a negative control.

Statistical Analysis. All results were reported as mean ± standard deviation (SD) of at least four independent measurements. The two-tailed Mann-Whitney *U*-test was used for statistical analysis, with the level of significance set at $p<0.05$. All statistical ana-

lyses were done using SPSS software package version 11.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Effect of Silymarin or Desferrioxamine on the Proliferation of Lymphocytes. The proliferation of PHA-activated lymphocytes exposed to silymarin or desferrioxamine was examined by BrdU incorporation assay. As shown in Figure1, silymarin at 20 µg/ml increased the proliferative response of lymphocytes in both normal and iron-treated lymphocytes, indicating no significant difference between the stimulatory effect of silymarin in normal and iron-treated conditions. The results also demonstrated that the proliferation of normal and iron-treated lymphocytes were significantly inhibited by 10 and 20 µg/ml of desferrioxamine. Strikingly, the inhibition of cell proliferation was less significant in iron-treated lymphocytes, indicating that excess iron partially saturated iron chelating activity of desferrioxamine, which is essential for its inhibitory effect on cell growth. However, the amount of iron loaded on lymphocytes was not enough for saturating the iron chelating activity of desferrioxamine and completely restoring cell proliferation to the normal level.

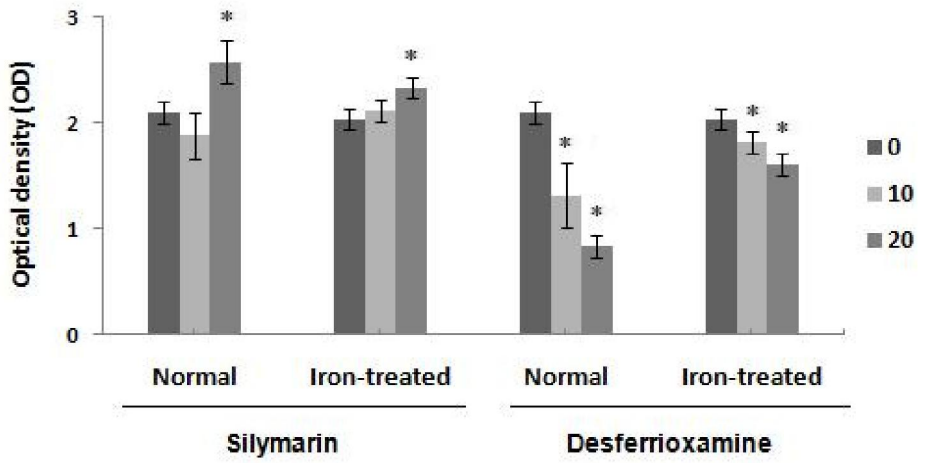

Figure 1. The effect of iron on the proliferation of PHA-activated lymphocytes exposed to 0, 10, and 20 µg/ml silymarin or desferrioxamine. Results were expressed as mean of optical density (OD) ± SD. Asterisks represent statistical significance when compared with corresponding control cells at zero concentration of silymarin or desferrioxamine ($p<0.05$). Iron-treated lymphocytes were considered as control cells for evaluating the influence of iron on the biological activity of silymarin and desferrioxamine.

Effect of Silymarin or Desferrioxamine on the Telomerase Activity of Lymphocytes. To gain an insight into the mechanism of growth modulation caused by desferrioxamine and silymarin, the telomerase activity of normal and iron-treated lymphocytes were evaluated using PCR-ELISA telomerase assay. Iron incubation markedly increased telomerase activity of lymphocytes compared to control cells under iron-free conditions (Figure2). However, the increase of telomerase activity in iron-loaded lymphocytes incubated with silymarin was not significant in comparison with cells exposed to silymarin under normal culture conditions. The results also revealed that desferrioxamine significantly inhibited telomerase activity of PHA-stimulated lymphocytes in both normal and

iron-treated conditions compared to those of corresponding control cells. However, the inhibitory effect of desferrioxamine on telomerase activity of lymphocytes was significantly abrogated by iron treatment (2.6±0.1 vs 3.1±0.1). On the other hand, iron treatment had no effect on the telomerase activity of lymphocytes incubated with silymarin (Figure2).

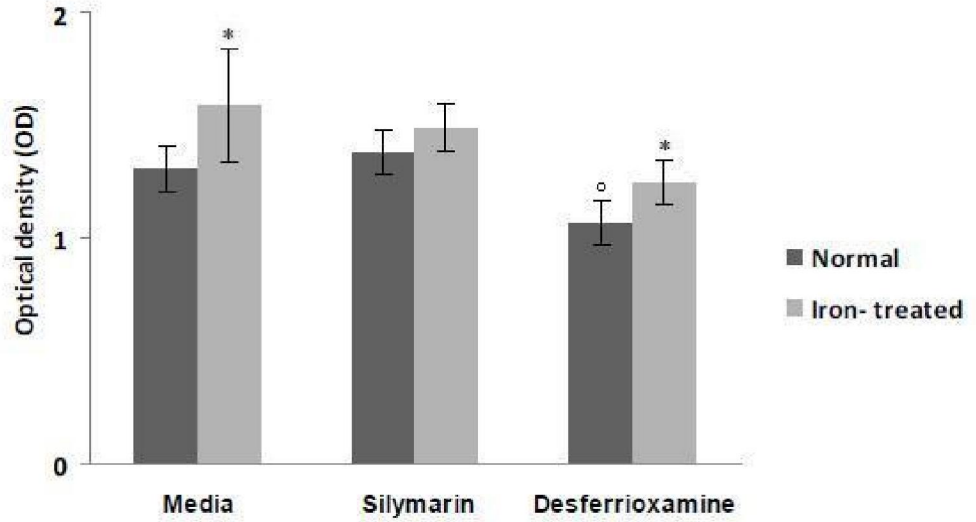

Figure 2. The effect of iron on telomerase activity of PHA-activated lymphocytes exposed to 20 µg/ml silymarin or desferrioxamine. Values are expressed as mean of optical density (OD) ± SD. Asterisks represent significant statistical difference between normal and iron-treated culture conditions. The circle represents significant difference between normal culture media and desferrioxamine ($p<0.05$).

DISCUSSION

Iron is an essential element for the proliferation of cells. Rapidly dividing cells need more iron, as shown by a high transferrin receptor expression on the cell membrane and increased cellular iron uptake. Desferrioxamine has been shown to be effective in inhibiting the growth of some neoplastic cells in vitro (1,2,13). Furthermore, it has been reported that desferrioxamine inhibited the proliferative response of lymphocytes induced by concanavalin A and pokeweed mitogen (14). In accordance with previous published data (3, 14, 15), the result of BrdU incorporation assay revealed that desferrioxamine significantly inhibits the proliferation of PHA-activated lymphocytes in a dose dependent manner. However, iron-treatment could partially abrogate antiproliferative effect of desferrioxamine on lymphocytes, probably due to the inadequate amount of iron loading to saturate desferrioxamine molecules completely. The explanation for this observation is that iron chelating effect of desferrioxamine may lead to the inhibition of the activity of ribonucleotide reductase, the enzyme responsible for deoxyribonucleotides synthesis, thereby resulting in the inhibition of DNA synthesis (4).

On the contrary, silymarin at 20 µg/ml increased proliferation of PHA-activated lymphocytes confirmed by BrdU assay. The results also demonstrated that silymarin had the same growth stimulatory effect on iron-treated lymphocytes. These results were in accordance with previous published articles on the biological effects of silymarin and silybin on lymphocytes and Jurkat cells (15-18). Silybin, the main component and the most biologically active component of silymarin, possesses antioxidant and iron chelating activities (10). This observation suggests that the growth stimulatory effect of silymarin is probably not related

to its iron chelating activity. Several studies have investigated the molecular mechanisms of the stimulatory and cytoprotective actions of silymarin. It has been shown that the enzymatic activity of DNA-dependent RNA-polymerase I is stimulated by silymarin, which subsequently accelerates the synthesis of ribosomal RNAs and also promotes the formation of complete ribosomes, leading to the increase of protein biosynthesis (19).

The results of the present study also revealed that the telomerase activity of activated lymphocytes was significantly increased in iron-treated cells. The increased hepatic telomerase activity has been demonstrated recently in a rat model of iron overload (20). Iron is a catalyst in the formation of reactive oxygen species (21), leading to oxidative tissue damage. G-rich sequences of telomeres are highly sensitive to oxidative damage, suggesting that oxidative damage induced by free radicals may also contribute to telomere shortening (22). As a result, it is reasonable to speculate that enhanced telomerase activity in iron-treated lymphocytes'probably caused by iron-mediated free radicals, may serve a protective function against DNA breakage and telomere shortening.

To support the above conclusion, results of the present study also revealed that desferrioxamine significantly inhibited telomerase activity of PHA-stimulated lymphocytes in both normal and iron-treated conditions compared to those of corresponding control cells. However, the inhibitory effect of desferrioxamine on telomerase activity of lymphocytes was significantly abrogated by iron treatment, suggesting that iron chelating activity of desferrioxamine may be responsible for preventing the formation of free radicals which may cause DNA breakage and telomere shortening.

Despite the significant increase of telomerase activity in iron-treated cells exposed to medium alone or to desferrioxamine, no significant change was detected in telomerase activity of iron-treated lymphocytes in the presence of silymarin. Several line of evidence demonstrated that oxidative stress accelerates telomere loss, whereas antioxidants decelerate telomere attrition (8, 22, 23). Silymarin as an antioxidant acts by scavenging pro-oxidant free radicals and increases the intracellular concentration of glutathione (17, 19). Another antioxidant property of silymarin may arise from the interactions between iron ions and silybin to produce complexes that prevent the participation of iron in free radical generating reactions (10). Therefore, silymarin may prevent DNA damage and telomere loss by chelating iron as well as scavenging free radicals in iron-treated lymphocytes. Consequently, it is reasonable to assume that no change in telomerase activity of iron-treated cells would occur in the presence of silymarin.

In conclusion, the results of present study demonstrate for the first time that iron is essential for proliferation and telomerase activity of PHA-activated lymphocytes. This was supported by the finding that iron deprivation by an iron chelator, desferrioxamine, could strongly inhibit the proliferation and telomerase activity of lymphocytes. Furthermore, the biological effects of silymarin on the proliferation and telomerase activity of normal and iron-treated lymphocytes were completely different from those of desferrioxamine. These findings may help elucidate the complex interactions between antioxidant and iron-chelating properties of silymarin. The molecular mechanisms underlying the immunomodulatory effects of silymarin await further investigation.

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