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ORIGINAL RESEARCH ARTICLE

The Telomerase Activity of Selenium-Induced Human Umbilical Cord Mesenchymal Stem Cells Is Associated with Different Levels of *c-Myc* and *p53* Expression

Leila Hosseinzadeh Anvar,[1,2] Saeid Hosseini-Asl,[2]
Mohammad Mohammadzadeh-Vardin,[1] and Mohsen Sagha[1]

Selenium—as a trace element—is nutritionally essential for humans. It prevents cancerous growth by inhibiting the telomerase activity but the mechanism involved in regulation of telomerase activity in normal telomerase-positive cells remains to be elucidated. Here, we find out whether the effect of sodium selenite and selenomethionine on telomerase activity in human umbilical cord-derived mesenchymal stem cells (hUCMSCs) is associated with different levels of *c-Myc* and *p53* expression. The use of different staining methods including ethidium bromide/acridine orange and DAPI in addition to telomeric repeat amplification protocol assay and real-time PCR indicated that different forms of selenium have opposite impacts on *c-Myc* and *p53* expressions in both hUCMSCs and AGS, a gastric adenocarcinoma cell line, as a positive control. Our findings suggest that the signaling pathways involved in the regulation of telomerase activity in malignant and normal telomerase-positive cell types are somewhat different, at least on the *c-Myc* and *P53* expression levels.

Keywords: telomerase activity, hUCMSCs, selenium, *c-Myc*, *P53*

Introduction

Selenium (Se)—as a trace element with antioxidant capacity—is nutritionally essential for many cellular processes in humans (Reszka *et al.*, 2012; Brigelius-Flohé and Maiorino, 2013). Some experimental studies suggest that selenium can inhibit the growth of cancer cells. This has led to widespread interest and claims that taking selenium supplements may prevent cancer. Since telomerase is an attractive target for cancer treatment and is required for essentially all tumors to undergo immortalization, interest focused in particular, on whether selenium compounds are able to reduce telomerase activity (Liu *et al.*, 2010). Some studies suggest that Se significantly changes the telomere length and cellular telomerase activity, but its mechanism remains to be elucidated (Shuang *et al.*, 2007; Wu *et al.*, 2009; Ferguson *et al.*, 2015).

Telomerase is composed of two main subunits: TERT and TERC. Unlike TERC, which is expressed in most cells and serves as a template for telomere replication, human telomerase reverse transcriptase (hTERT), a catalytic subunit of the human telomerase, is an effective factor in regulation of telomerase activity (Sandin and Rhodes, 2014). Recent data demonstrate a close correlation between *hTERT* expression and telomerase activity in human tumors (Rao *et al.*, 2011; Lue and Autexier, 2012; Wong *et al.*, 2014). Thus, the *hTERT* may be an excellent candidate for cancer therapy (Holysz *et al.*, 2013), and it was found that the promoter of *hTERT* has binding sites for c-Myc and other transcription factors (Kyo *et al.*, 2008; Calado, *et al.*, 2009; Zhao *et al.*, 2014; Lewis and Tollefsbol, 2016). In addition, some trace elements like selenium can regulate its expression. There is evidence reporting a link between overexpression of *P53* as a transcription factor involved in apoptotic pathways and *c-Myc* at a relatively high dose of selenium in rat hepatocyte (Yu *et al.*, 2009). Some researchers believed that both *c-Myc* and *p53* overexpression—mediated by oxidative stress—may represent a key role in selenium-induced apoptosis (K, 2006; Yu *et al.*, 2006a, 2006b; Rudolf, 2008). Various investigations also suggested that the different forms of selenium have their own unique sets of abilities for telomerase activity regulation in different cell types at the upstream or downstream levels (Chen *et al.*, 2007; Yu *et al.*, 2009; Zarghami, *et al.*, 2011). It has been shown that the sodium selenite activates *p53* at the transcriptional level in prostatic cancer cells to induce cell death (Sarveswaran *et al.*, 2010). The downregulation of *c-Myc* has also been observed in colorectal carcinoma cell line HCT116 following selenomethionine treatment (Goulet *et al.*, 2007). Moreover, both organic and inorganic forms of selenium promoted HL-60 cell cycle progression through overexpression of some cell cycle-

[1]Research Laboratory for Embryology and Stem Cells, Department of Anatomical Sciences and Pathology, Faculty of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran.
[2]Laboratory of Medical Genetics, Imam Khomeini Hospital, Ardabil University of Medical Sciences, Ardabil, Iran.

related genes, especially *c-Myc* (Zeng *et al.*, 2002). Although some old studies have indicated the relationship between telomerase activity and *c-Myc/P53*, especially in selenium-exposed cancerous cells, the mechanism by which telomerase regulation is associated with *c-Myc* and *P53* has not been fully understood yet. There is no direct report indicating the regulation of telomerase function by these transcription factors in normal telomerase-positive cells when exposed to selenium compounds. Therefore, in this study, we aimed to use the human umbilical cord-derived mesenchymal stem cell (hUCMSC) as a telomerase-positive fetal stem cell model to determine the impact of different selenium forms on *c-Myc* and *p53* expression profiles and their association with telomerase activity. We found that sodium selenite upregulates *c-Myc/p53* expression and induces the higher telomerase activity in hUCMSCs, but selenomethionine shows an opposite effect and decreases *c-Myc/p53* expression and telomerase activity.

Materials and Methods

Cell culture

Human Wharton's jelly-derived mesenchymal stem cells (the hUCMSCs) were isolated and cultured as previously described (Saraee *et al.*, 2014). In brief, after obtaining fresh umbilical cord from full-term neonates (in accordance with the ARUMS ethical committee's considerations), they were dissected into small pieces and transferred to a T-25 flask containing DMEM medium (ATOCEL; ATCDL-891) supplement with 20% fetal bovine serum (FBS, Gibco;10270-098) and 2% Pen/Strep/AmphoB (ATOCEL, ATRB-010) and finally incubated at 37°C and 5% CO2. By 20 days postculturing, the remaining explants were removed and the growing hUCMSCs were subcultured in $DMEM^{LG}$ with 10% FBS. This stage was denoted as Passage 0. Subsequent passages of the hUCMSCs were obtained by sequential subculturing. The AGS, a gastric adenocarcinoma cell line as a human gastric cancer cell model was expanded in RPMI1640 medium (ATOCEL, ATCR-885) supplement with 10% FBS and 1% Pen/Strep/AmphoB at 37°C under a humidified atmosphere of 5% CO2.

Experimental design

As shown in Figure 1, the hUCMSCs at a density of 1000 cells/well were seeded in 96-well tissue culture plates and treated with different concentrations of sodium selenite (NaSel, Sigma; S5261), ranging from 1 to 80 μM dissolved in phosphate-buffered saline [PBS]) and selenomethionine (SeMet, Sigma; S3132) at a range of 0.5–2000 μM. Untreated cells were considered a control group.

Cell viability tests

To investigate the viability rate of hUMCSCs at different doses of NaSel and SeMet and to obtain the optimum dose at which the cells are less sensitive to the cytotoxicity from selenium compounds, MTT assay, DAPI, and ethidium bromide/acridine orange (EB/AO) staining methods were used as previously described (Saraee *et al.*, 2014). For the quantification of the cell death rate, 15 microscopic fields were chosen at ×20 magnification and the photos were taken with an inverted fluorescence microscope (Olympus IX71, Japan) equipped with a digital camera (Olympus; DP71).

Flowcytometry analysis

Characterization of hUCMSCs by flowcytometry (Partec, CyFlow) has been done as previously described (Saraee *et al.*, 2014) by using different positive (CD44-FITC, CD73-PE, CD90-PE, and CD105-FITC) and negative (CD34-PE and CD45-FITC) (BD Pharmingen, England) stemness markers. To analyze apoptosis, DAPI (40,6-diamidino-2-phenylindole, Gerbu,1050) staining were used. For this purpose, the hUCMSCs were precultured for 72 h and treated with 3, 10, and 20 μM of NaSel for 24 h and 500 μM SeMet for 72 h. Then, the cells were detached from the culture dish by 0.05% trypsin/EDTA, centrifuged at 12,000 rpm for 5 min, and exposed to DAPI (1 μg/mL) for 30 min. Finally, to demonstrate the percentage of apoptotic cells, cell cycle analysis was performed.

TRAP assay

Telomeric repeat amplification protocol (TRAP assay) is a qualitative and PCR-based telomerase assay that can evaluate the telomerase activity. A pellet of 10^5 cultured cells from both untreated and treated groups was washed with PBS and homogenized in 200 μL of ice-cold Chaps lysis buffer. After incubating at −20°C for 30 min, the cell lysate was centrifuged at −4°C and the resultant supernatant was stored for subsequent usage or aspirated to define the protein concentration by Bradford protein assay kit (Bio-Rad, B6916). In the next step, 1 μg of protein was added to a TRAP master mix containing TS primer (in a final volume of 45 μL) and incubated at room temperature for 30 min to extend the substrate by telomerase and inactivated at 95°C for 5 min. Then, 5 μL of a mixture of 2.5 U Taq DNA polymerase, 50 ng ACX, 50 ng NT, and 1.3×10^{-9} ng TSNT primers were added to the extended product and finally subjected to PCR condition as follows: 94°C for 30 s, 52°C for 60 s, and 72°C for 60 s. Then, the PCR product was electrophoresed on 10% nondenaturing

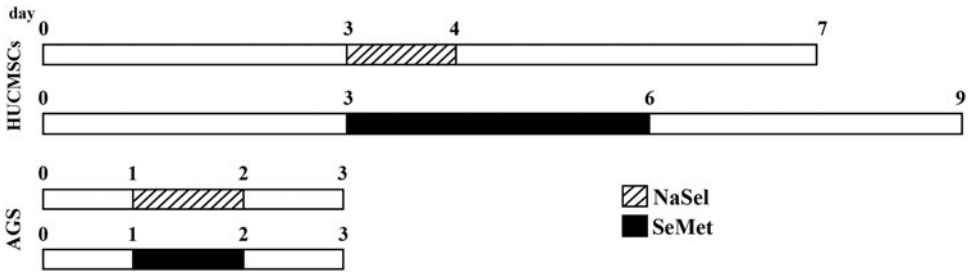

FIG. 1. The experimental design for AGS, a gastric adenocarcinoma cell line and hUCMSCs culture and treatment with different concentrations of NaSel and SeMet. Pretreatment time for each cell type has been calculated based on the cell specific-doubling time. hUCMSC, human umbilical cord-derived mesenchymal stem cell.

polyacrylamide gels. In this assay, the AGS cell line was used as a positive control showing high telomerase activity.

Quantitative real-time TRAP assay

For quantitative analysis of the telomerase activity by real-time PCR (qRT-TRAP Assay), 1 μL of the extracted protein was added to 24 μL solution containing 1× SYBR Green buffer, 50 ng TS, and 50 ng ACX primers. After incubation at room temperature for 30 min, the amplified product was run in a thermal cycler (Roche; 05815916001) for 40 cycles at 95°C for 10 min (to activate the hot-start Taq polymerase in the master mix), 95°C for 30 min, and 60°C for 60 s.

RNA extraction and complementary DNA synthesis

Total RNA was isolated from the cells by Biozol (BioFlux; BSC51 M1) reagent in accordance with the manufacturer's protocol. To eliminate any DNA contamination, the extracted RNA was treated with the DNase-I enzyme (Cinnaclon, PR891627C). Then, the complementary DNA (cDNA) from 1 μg DNase-I digested RNA was synthesized by using RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentase, K1622). The samples were stored at −20°C until analysis via quantitative real-time polymerase chain reaction (qRT-PCR).

Quantitative real-time PCR

Quantitative real-time PCR was performed by light cycler real-time PCR detection system (Roche; 05815916001). Briefly, 1 μL of each cDNA template was added to a mixture containing 1× SYBR Green buffer (EURx; E0402-01) and 400 nM of each forward and reverse primer. The primer sequences and the PCR product sizes were shown in Table 1. Real-time PCR reactions were carried out as follows: 10 min at 95°C for one cycle; 30 s at 95°C, 60 s at 60°C for 40 cycles. Then, the melting curve for each PCR product was analyzed. Fold changes in gene expression for treated and untreated groups were determined by using the $2^{-\Delta\Delta CT}$ method and the housekeeping gene, GAPDH as an internal control. The real-time PCR reactions were done in triplicate and again, AGS cell line was used as a positive control group expressing the desired genes.

Statistical analysis

The experimental assays for quantification were carried out at least three independent repeats and the resulted data were expressed as mean ± standard error of mean. Then, based on the collected data, two-independent Student's *T*-test or One-way ANOVA followed by Tukey's *post-hoc* multiple group comparison test were used to statistically analyze different groups. A difference between groups was considered statistically significant if $p < 0.05$.

Results

Effect of NaSel and SeMet on hUCMSCs morphology

Following culture and expansion of the hUCMSCs up to P2 and characterizing them by several mesenchymal stem cell markers, they were exposed to the selenium compounds, that is, NaSel and SeMet at different doses to find whether these selenium agents have any effects on cell morphology and viability. As depicted in Figure 2, our results clearly illustrated that the hUCMSCs expressed the mesenchymal stem cell's markers CD44 (69.22%), CD73 (99.86%), CD90 (98.35%), and CD105 (74.05%) but very few cells expressed the hematopoietic stem cell's markers CD34 (0.44%) and CD45 (7.51%). Moreover, NaSel at 3 μM altered the cell morphology to a slender shape but most of them were alive. However, treatment of hUCMSCs with NaSel at the higher concentration (10 μM) widely induced cell death, so that many cells represented a round and marginal bright configuration, suggesting many cells had undergone to e apoptosis. In contrast, hUCMSCs exposed to SeMet at different doses (0.5 and 1 mM) showed no obvious changes in cell morphology and represented a resistance even at the higher doses.

Effect of NaSel and SeMet on cell viability

To investigate the viability rate of hUCMSCs when treated with different doses of selenium compounds and to find the optimum concentrations at which the cells are more stable and less sensitive to NaSel and SeMet cytotoxicity, MTT assay was used. Our results indicated that mean viability rate of the cells—when exposed to the different concentrations of NaSel for 24 h—significantly decreased from 78.3% ± 2.95% and 55.93% ± 5.08% at 3 and 10 μM to 6.9% ± 1.81% and 1.48% ± 0.3% at 20 and 80 μM, respectively (Fig. 3A, $p < 0.01$). The hUCMSCs treated with SeMet for 72 h from 0.5 to 2000 μM showed a different story in which no significant changes in the cell viability rate was obtained. According to our data, we selected 3 μM NaSel and 500 μM SeMet as the optimum amounts at which the cells were resistant to cell death and about 80% of them were alive.

To check the cytoplasmic and nuclear changes in hUCMSCs when exposed to the aforementioned optimum quantities of the selenium agents, the treated cells were stained with EB/AO. As shown in Figure 4, tiny blebbed cytoplasmic changes and cell shrinkage along with nuclear fragmentations were seen in the

Table 1. Primer Sequences and PCR Product Length Used for qRT-PCR

Gene	*Sequence (5′-3′)*		*Length (bp)*
c-Myc	Forward	GGCTCCTGGCAAAAGGTCA	113
	Reverse	AGTTGTGCTGATGTGTGGAGA	
P53	Forward	TTTGCGTGTGGAGTATTTGG	99
	Reverse	TGGATGGTGGTACAGTCAGAG	
hTERT	Forward	CTTTTATGTCACGGAGACCAC	101
	Reverse	TCAAGTGCTGTCTGATTCCAATG	
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC	226
	Reverse	AAGATGGTGATGGGATTTC	

hTERT, human telomerase reverse transcriptase; qRT-PCR, quantitative real-time polymerase chain reaction.

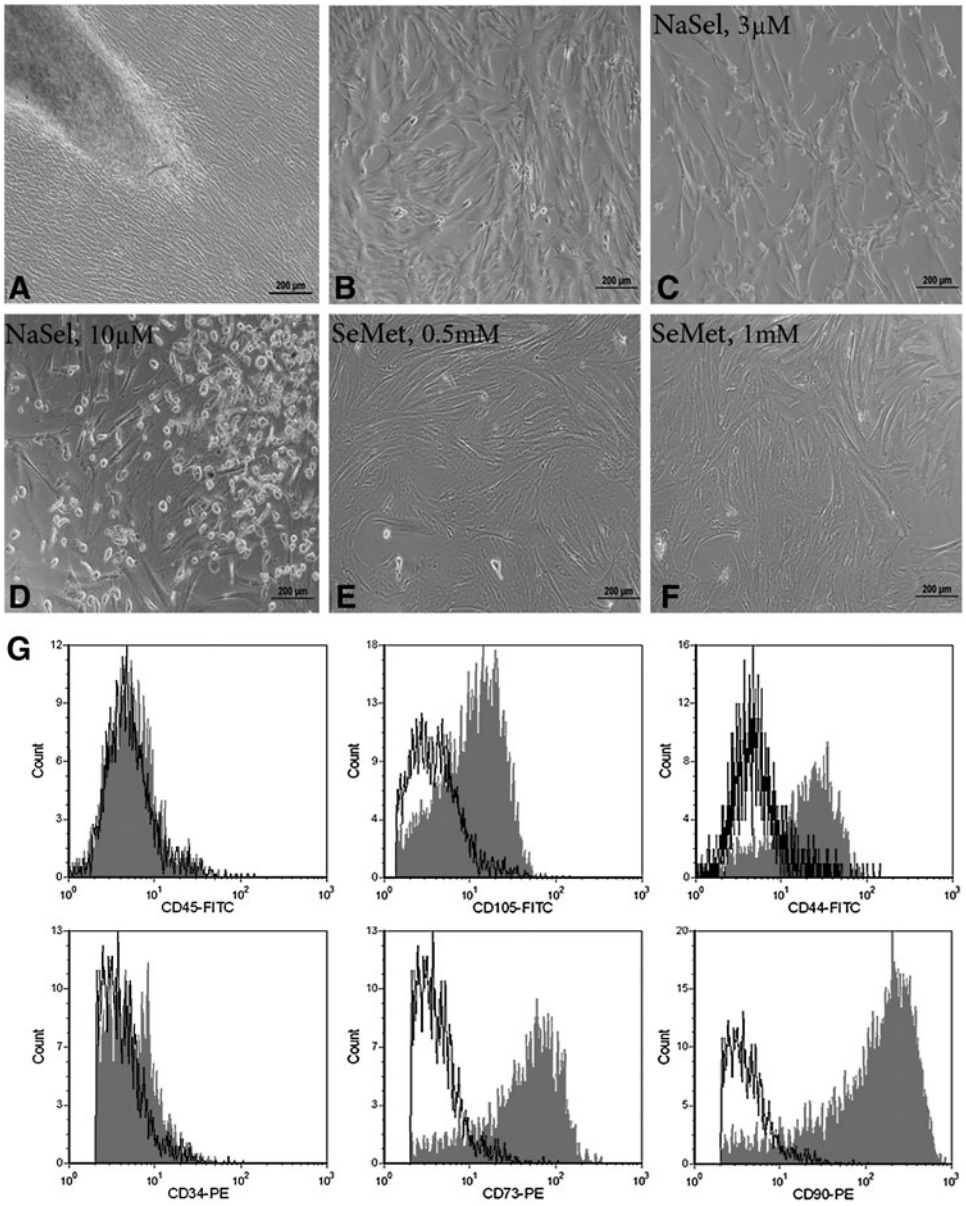

FIG. 2. Treatment of the hUCMSCs with different concentrations of selenium compounds. **(A)** Umbilical cord-derived Wharton's jelly explants were plated for 20 days and **(B)** a growing number of dissociated cells represented a fibroblast-like and endothelial-like cell morphology at P2. **(C)** Photomicrograph of 3 μM NaSel-treated hUCMSCs cells, resembling the fibroblast-like cells with slender processes and **(D)** the same cells when exposed to the higher dose (10 μM), inducing apoptosis in a great number of the cells **(E)** The hUCMSCs appeared with no considerable changes in their morphology at 0.5 and **(F)** 1 mM SeMet even after 72 h culture. **(G)** Different positive (CD73, CD90, CD44, CD105) and negative (CD34 and CD45) markers representing stemness of hUCMSCs.

cells treated with 3 μM NaSel, but these morphological alterations enhanced in the cells exposed to 10 μM, suggesting early and late apoptosis and necrosis injury occurred at the higher quantity (Fig. A1–D2). These observations were confirmed by DAPI staining quantification through which the cell death frequency significantly increased from 20.38% ± 3.79% at 3 μM to 34.38% ± 4.46% at 10 μM NaSel ($p < 0.01$, Fig. 4E). Furthermore, when the cells treated with 500 μM SeMet, only% 3.32 ± 0.66 of them showed apoptosis compared to 3.04% ± 0.98% of cells in control group ($p > 0.05$) and EB/AO staining demonstrated no apparent difference between the treated and untreated cells. Analysis of DAPI- stained apoptotic cells and cell cycle by flowcytometry clearly showed that NaSel at the higher doses (10 and 20 μM) induced apoptosis in 55.23% and 62.45% of cells in comparison with 3 μM concentration at which 39.15% of cells underwent apoptosis. As expected, just 8.48% of hUCMSCs indicated cell death in the SeMet (500 μM)-treated group (Fig. 4F).

Effect of NaSel and SeMet on telomerase activity in the hUCMSCs

To investigate the effect of NaSel and SeMet on telomerase activity, TRAP assay and quantitative real-time TRAP assay were performed (Fig. 5). Since 3 μM NaSel and 500 μM SeMet showed very low cytotoxic effects on hUCMSCc, in the remaining experiments they were considered as the optimum doses influencing the cells. As expected, by using the TRAP assay, the hUCMSCs obviously showed high telomerase activity, which upregulated upon treating with NaSel and downregulated when the cells exposed to SeMet. To quantify the Ct at which the treated cells indicate alterations in telomerase activity, qRT-TRAP assay calculated 22.25 ± 0.9 and 31.99 ± 1.01 Ct in NaSel and SeMet-exposed cells, respectively. These data were significantly different from the Ct for the untreated cells (27.05 ± 0.58, $p < 0.05$) (Fig. 5B).

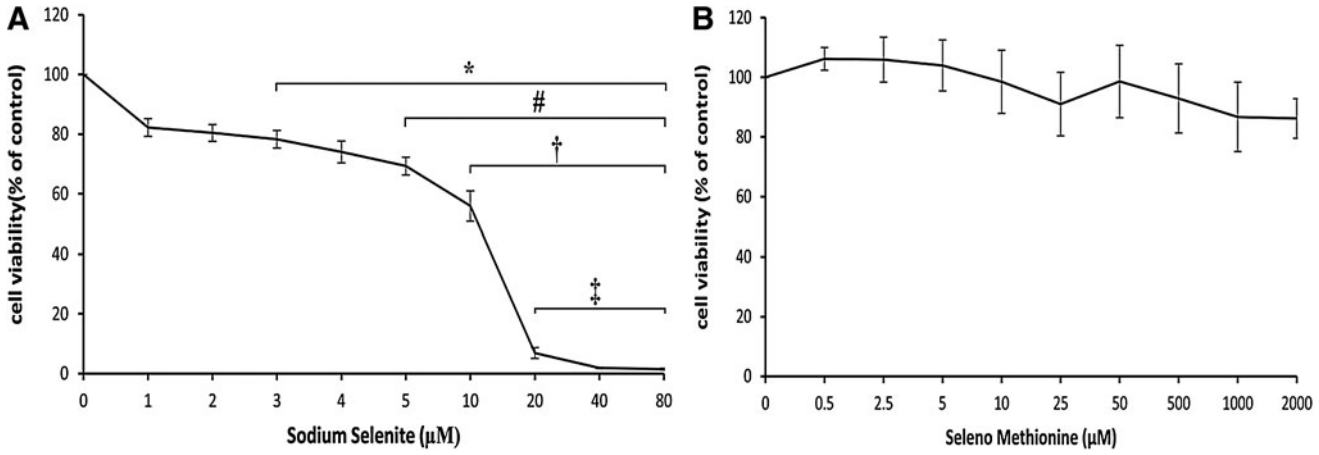

FIG. 3. Effect of selenium supplementation on the cell viability rate. **(A)** The hUCMSCs were incubated for 24 h with increasing concentrations of sodium selenite (up to 80 μM). **(B)** The same cells when exposed to different concentrations of selenomethionine (up to 2000 μM). (*$p < 0.01$ vs. Control group, $^{\sharp}p < 0.01$ vs. 1 & 2 μM, $^{\dagger}p < 0.01$ vs. 3 μM and $^{\ddagger}p < 0.01$ vs. 4,5 & 10 μM).

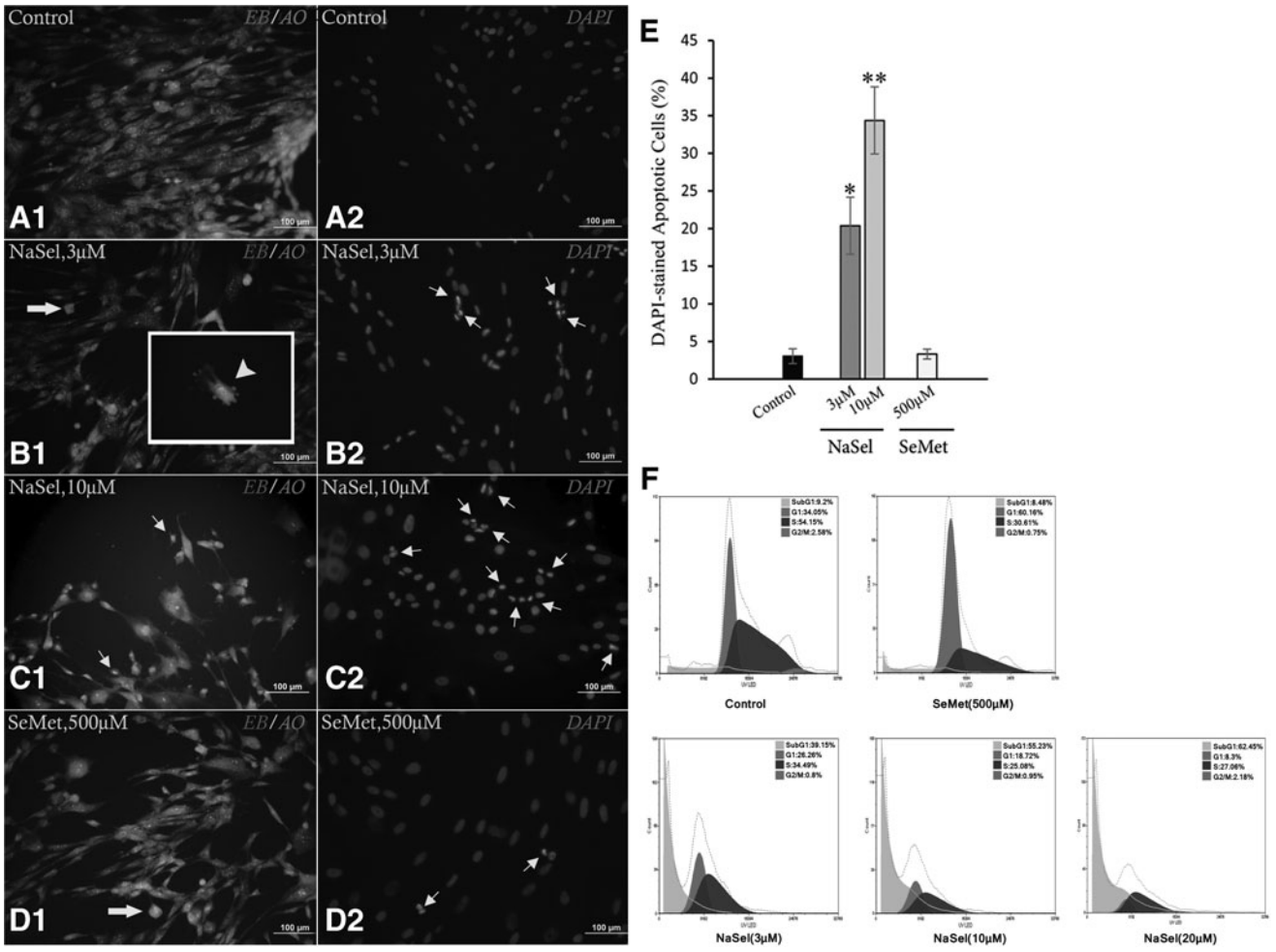

FIG. 4. Assessment of the hUCMSCs cytoplasmic and nuclear alterations by EB/AO and DAPI staining methods. **(A1, A2)** Untreated hUCMSCs in the control group. **(B1–C2)** The hUCMSCs at different stage of apoptotsis after being treated with 3 and 10 μM sodium selenite. A cell shown in *square* represents the cytoplasmic blebbing induced by NaSel **(D1, D2)** The treated hUCMSCs with selenomethionine at 500 μM. The *arrowhead* represents the early apoptosis, *thin arrows* show the late apoptosis, and the *thick arrows* show the necrotic cell. **(E)** Quantification of the DAPI-stained apoptotic cells in different groups represents the higher percentage of cell death in 10 μM sodium selenite than the other groups (*$p < 0.05$, **$p < 0.01$ vs. control group). **(F)** Cell cycle analysis of NaSel-treated hUCMSCs at different doses (3, 10, and 20 μM) and SeMet-treated cells at 500 μM concentration. SubG1 denotes the rate of apoptosis. EB/AO, ethidium bromide/acridine orange.

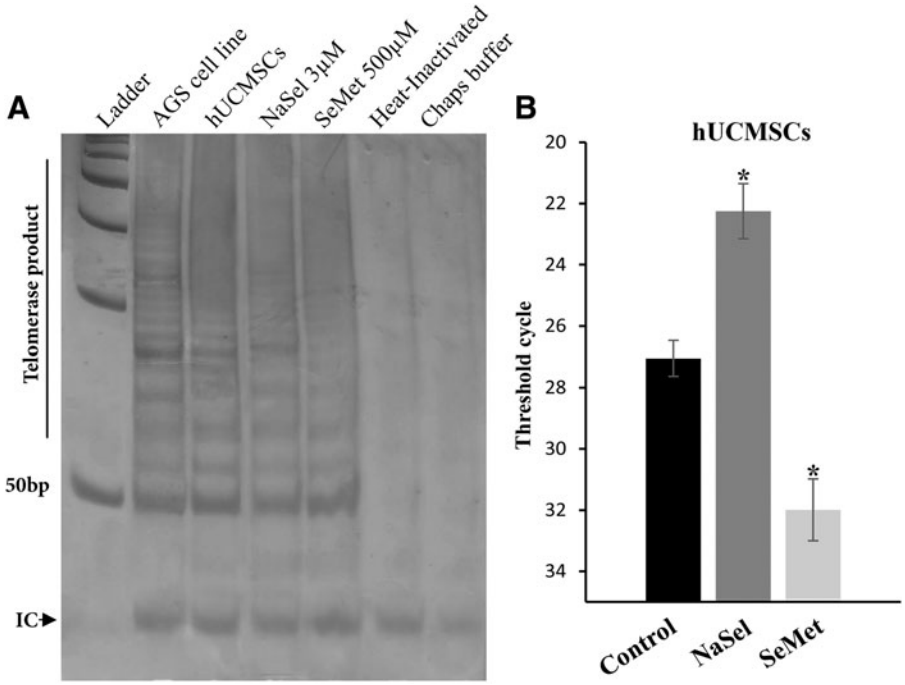

FIG. 5. Effect of selenium compounds on the telomerase activity in hUCMSCs. **(A)** TRAP assessment of telomerase activity in NaSel- and SeMet-treated hUCMSCs. AGS cell line was selected as a positive group indicating the high telomerase activity. Heat-inactivated and Chaps lysis buffer were represented as the negative controls. **(B)** Quantification of the telomerase activity in the treated and untreated cells by qRT-TRAP assay. IC denotes the internal control (*$p < 0.05$ vs. control group). TRAP, telomeric repeat amplification protocol.

Effect of NaSel and SeMet on c-Myc and P53 mRNA expression in the hUCMSCs

It is known that telomerase activity is regulated by *c-Myc* and *P53*. Moreover, exposure to different forms of selenium appears to have controversial effects on the genes noted. To address this possibility, as indicated by Figure 6, real-time PCR analysis obviously represented that *c-Myc* expression in the NaSel-treated hUCMSCs at the mRNA level significantly increased to 1.83 ± 0.29-fold changes compared to the untreated cells ($p < 0.05$). In contrast, its expression in NaSel-treated AGS was downregulated to 0.26 ± 0.21 compared to the untreated AGS cells ($p < 0.05$). Similar results were obtained for p53 expression level when both the cells were treated with NaSel (2.13 ± 0.21-fold changes for the hUCMSCs and 0.28 ± 0.14 for the treated AGS cells, [$p < 0.05$ vs. both control groups]). The organic form of the selenium revealed the opposite impacts on *c-Myc* and *P53* expression in the hUCMSCs. The expression of *c-Myc* in SeMet-treated hUCMSCs was found to have changes around 0.73 ± 0.13-fold, but was significantly downregulated to 0.33 ± 0.13 in SeMet-exposed AGS cells in comparison with the untreated cells ($p < 0.05$). No distinct alteration in p53 expression was detected in SeMet-exposed hUCMSCs (0.96 ± 0.18), but p53 expression again decreased to 0.2 ± 0.02-fold changes in SeMet-treated AGS cell.

Discussion

In this study, we presented a relationship between telomerase activity and levels of *c-Myc/p53* expression in the hUCMSCs as the fetal stem cells, which are similar to cancerous cells, are telomerase-positive cells. We also reported evidence that NaSel at physiological concentration has an opposite effect on hUCMSCs compared to AGS cancer cell line to enhance telomerase activity through upregulating *c-Myc/p53* expression but the effect of SeMet on both cell types appeared to be the same. While some reports suggest that sodium selenite can extend the telomere length and increased telomerase activity in hepatic cell line and yeast cells (Liu *et al.*, 2004; Yu *et al.*, 2009) in contrast, studies on breast

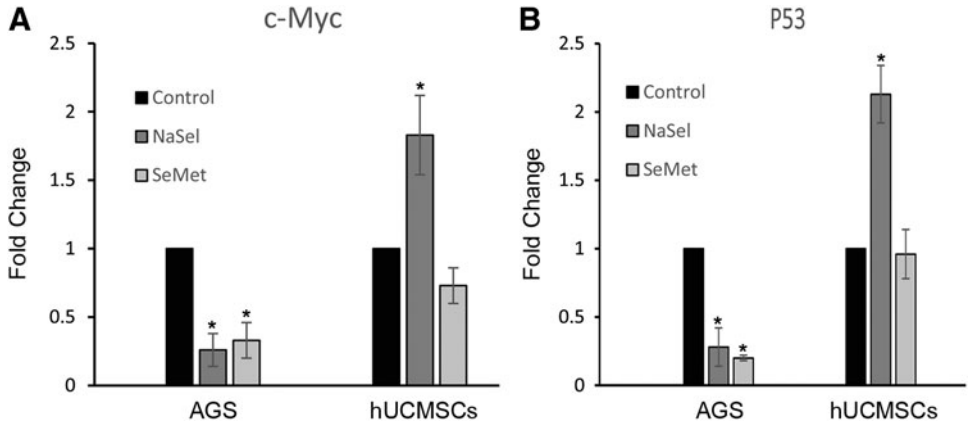

FIG. 6. Effects of NaSel and SeMet on c-Myc and p53 mRNA expression in hUCMSCs and AGS cell line. Real-time PCR analysis of indicated genes was performed. All results were normalized to GAPDH expression level (*$p < 0.05$ vs. control group).

cancer cells and human bronchial cells clearly demonstrate that sodium selenite represents the opposite effects to diminish telomerase activity (Chen *et al.*, 2007; Zarghami, 2011). However, what are the distinct molecular mechanisms involved in the regulation of telomerase activity in the selenium-induced cells? This remains to be understood. So, in the remaining study we tried to find whether telomerase activity is related to *c-Myc* and *P53* expression levels.

Sodium selenite-exposed hUCMSCs apparently revealed the higher telomerase activity and this function was found to be associated with overexpression of *c-Myc* and *p53* compared to the untreated cells. We also noticed that these results were completely different compared to the NaSel-induced AGS cells in which selenium downregulated telomerase activity and c-Myc and P53, suggesting NaSel inducing ability on telomerase activity is fully dependent on the cell type. SeMet-induced hUCMSCs showed a completely different story so that *c-Myc* and *p53* were underexpressed, and this downregulation was accompanied by reduction in telomerase activity. Hence, these findings were similar to what happened in AGS cells, proposing the idea that organoselenium may show an anti-telomerase function, independent of the cell type. Chi-Hwa *et al.*, have reported that *c-Myc* inactivation—either *in vitro* or *in vivo*—results in cellular senescence, suggesting the inactivation of telomerase (Chi-Hwa *et al.*, 2007). However, using an inducible *c-Myc* overexpression, Drissi *et al.* have made an opposite system of observations in human leukaemia cells, where *c-Myc* activation leads to apoptosis, but no alterations in TERT levels or telomerase activity were evident (Drissi *et al.*, 2001). Another study in rat hepatocytes also confirmed our results that NaSel increases the cellular telomerase activity and *hTERT/c-Myc/p53* gene expression level (Yu *et al.*, 2009). Moreover, it has been shown that exposure of leukemic cells to antisense *c-Myc* also resulted in the inhibition of telomerase activity (Fujimoto and Takahashi, 1997).

The telomerase-positive cells should express *hTERT* as a functional subunit that can be regulated by *c-Myc* (Wick *et al.*, 1999). To evaluate any relationship between hTERT and *c-Myc* with telomerase activity, we measured *hTERT* expression level in selenium-induced AGS and found that both NaSel and SeMet significantly diminished *hTERT* expression up to 7- and 23-fold in comparison with untreated cells. However, we could not detect any *hTERT* expression in hUCMSCs in untreated and selenium-exposed cells. No *hTERT* mRNA expression in hUCMSCs may reflect on the existence of under-detectable amounts of hTERT mRNA in these cells but this level is sufficient for initiation or acceleration of telomerase activity. Using TaqMan-based real-time PCR, Wang *et al.* found that *hTERT* was expressed in hUCMSCs at a very low level (Wang *et al.*, 2014).

In conclusion, the telomerase activity is related to *hTERT*, *c-Myc*, and *p53* expression levels and selenium compounds have different telomerase activity effects on both the hUCMSCs and gastric cancer cells, but the exact molecular signaling transduction pathway promoting these diverse impacts remains to be elucidated further.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Mohsen Sagha, PhD
Department of Anatomical Sciences and Pathology
Faculty of Medicine
Ardabil University of Medical Sciences
University Street
Ardabil province
Ardabil 56189-53141
Iran

E-mail: m.sagha@arums.ac.ir

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