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Biphasic Dose–Response and Hormetic Effects of Stress Hormone Hydrocortisone on Telomerase-Immortalized Human Bone Marrow Stem Cells In Vitro

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Abstract

Although high levels of stress hormones are associated with well-known negative health outcomes, their low levels can have health-promoting effects by virtue of the phenomenon of mild stress-induced hormesis. We have studied the effects of a wide range (between 100 nmol/L and 150 μmol/L) of hydrocortisone (HC) on human bone marrow stem cells in vitro. Telomerase-immortalized human mesenchymal stem cells (hTERT-MSCs) were exposed to various doses of HC for different durations (1-6 days) and analyzed for survival and metabolic activity by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, for cell migratory ability by a wound-healing assay and for osteoblastic and adipogenic differentiation abilities in vitro. Our findings indicate that hTERT-MSCs exposed to HC resulted in a biphasic hormetic dose–response in some measures but not all. Although the mitochondrial and metabolic MTT activity assay clearly showed low-level stimulatory (between 0.1 and 1 μmol/L) and high-level inhibitory effects (from about 10 μmol/L onward), the cytostatic and differentiation-inducing effects were mostly linear at concentrations between 1 and 100 μmol/L. Further long-term studies will elucidate whether chronic or intermittent exposure of human cells to stress hormones has physiologically beneficial hormetic effects.

Keywords

hydrocortisone, hTERT-MSC, hormesis, differentiation, wound healing

Introduction

The stress hormone cortisol or hydrocortisone (HC) is the main glucocorticoid (GC), and a biological mediator of psychological stress, secreted by the adrenal gland and controlled by the hypothalamic–pituitary–adrenal axis via negative feedback.[1] Normal human blood cortisol levels follow a circadian rhythm with maximum secretion during the first few hours of the morning, decline during the daytime, and reaching the lowest levels by midnight (range 193-772 nmol/L).[2-4] A significant variation from this range over a longer term can lead to either a hypercortisol-related disease Cushing syndrome (CS) or hypocortisol-related condition, Addison disease.[2-4] In normal healthy condition, most of the circulating cortisol in the blood is in a bound form with albumin and transcortin (also known as corticosteroid-binding globulin or serpin A6 protein), and only about 5% circulates as unbound cortisol in a bioactive form.[3] Cortisol plays a major role in several biological processes, including regulation of energy metabolism, of levels of various metabolic hormones, of stress responses, of cell proliferation and differentiation, as well as memory mapping and maintenance of cognitive functions.[3]

Although high and chronically elevated levels of stress hormones are well known to be associated with a range of physical and mental problems,[5] little is known about the possible biphasic dose–response and hormetic effects of this stress hormone. The earliest reports on the positive effects of cortisone on extending the proliferative life span of

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human fibroblasts demonstrated that a chronic exposure of different human fibroblast strains to either 6.89 or 14 μmol/L cortisone increased their life span in vitro by 17% to 40%, depending on the cell strain.[6,7] There are also reports that skin fibroblasts isolated from the patients with CS, who have chronically elevated levels of GC, had longer proliferative life span in vitro.[8] This observation was further extended to show that when CS fibroblasts were removed from the high-GC milieu in vivo and cultured in vitro under standard conditions, they expressed an "anabolic" phenotype, in terms of restoring their ability for collagen synthesis, secreting reduced levels of metalloproteases (matrix metalloproteinase (MMP) 1 and MMP-2), and had an increased proliferative capacity and contractility.[9,10] Furthermore, these cells responded better to exogenous stress by producing significantly higher levels of heat-shock protein 70.[9,10] These data suggest that prior exposure to elevated GC concentrations is not associated with persisting adverse effects on fibroblasts and may also have a beneficial outcome in some aspects of cell physiology, including longevity in vitro.[11,12] Similarly, positive and physiologically beneficial effects of cortisol (2.5 μmol/L) in suppressing some aspects of the senescence-associated secretory phenotype in human fibroblasts have been reported.[13]

However, almost all studies cited above have generally used only 1 or 2 doses of cortisol in their experimental setup, which covers a very limited range. Since the theory of hormesis essentially involves the biphasic dose–response as one of its salient features,[14-16] we have tested a wide range (more than 1500-fold; from 100 nmol/L to 150 μmol/L) of cortisone levels for their immediate- and mid-term effects on the survival, proliferation, wound healing, and differentiation abilities of human bone marrow stem cells in vitro. Our results, as reported below, indicate that cortisone demonstrates a biphasic dose–response in which low levels (until about 1 μmol/L) induce a variety of positive effects before progressively becoming harmful for human stem cells in vitro. These observations have implications for the role of stress hormones in health maintenance and its modulation by hormetic approaches.

Materials and Methods

Chemicals and Kits

1α,25-Dihydroxyvitamin-D3 (vitamin D; calcitrol; Sigma #D1530), β-glycerophosphate (CALBIOCHEM #35675), Alizarin Red S (Sigma-Aldrich #A5533), alkaline buffer solution 1.5 mol/L (Sigma #A9226), alkaline phosphatase (ALP; Sigma #P-5931), Dulbecco's modified Eagle's medium (DMEM; BioWhitaker #BE12-604F), HC (Sigma #H0888), L-ascorbic acid (Sigma #A4544), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma #M2128), p-nitrophenol phosphate (pNPP; Sigma-Aldrich #N3002), p-nitrophenol standard solution 10 mmol/L (Sigma #N7660), protein assay detection kit (BIO-RAD #500-0112), and senescence cells staining kit (Sigma #CS0030).

Cell Culture

Telomerase-immortalized human mesenchymal stem cell lines, designated hTERT-MSCs, was generated by a stable retroviral transfection of human bone marrow stem cells with catalytic subunit of telomerase with reverse transcriptase activity, as previously described in detail.[17] This cell line exhibits unlimited proliferative ability while maintaining normal cellular functions, including differentiation and bone forming capacity in vitro and in vivo.[18] Cells were routinely cultured as described earlier,[17] using DMEM containing 10% (vol/vol) bovine fetal calf serum, 100 U/mL penicillin, 100 μL/mL streptomycin, and incubated at 37°C, 5% CO_2, 95% humidity. For subculturing and serial passaging, the cell cultures at near-confluent state were split using trypsin/EDTA. All experiments were performed at passage levels between 52 and 74, which is a rapidly proliferating stage without any drift into the potential tumorigenic mode.[19,20] Cell numbers were counted in trypsinized cell suspensions using a Beckman Coulter Counter, and the senescence-associated β-galactosidase (SABG) assay[21] was performed using the histochemical staining kit from Sigma-Aldrich.

Stock solution of HC (150 μmol/L; molecular weight 362.46) was freshly prepared for each assay by dissolving HC in a small volume of 100% ethanol, followed by the addition of the appropriate volume of complete DMEM and filtration through a 0.22-ipm filter (Millipore). The final concentration of ethanol in the HC solution was less than 1%.

Survival and Metabolic Activity

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. The biochemical assay that measures the metabolic activity and is also a measure of cell survival[22] was performed as described previously.[23-25] Briefly, hTERT-MSCs were seeded in 96-well plates at a density of either 3600 or 8000 cells/well for 1-day or 3-day experiments, respectively. After 24 hours of cell attachment, cells were exposed to a wide range of HC concentrations (from 100 nmol/L to 150 μmol/L) by replacing the old DMEM with fresh medium containing HC and then cells were incubated at normal culturing conditions. After experimental duration (1-3 days), the medium was carefully replaced with 200 μL media containing MTT reagent (0.5 mg/mL) and cells were incubated for 4 hours. Formazan crystals formed inside the cells were extracted with 100 μL of dimethyl sulfoxide for 15 minutes, and the absorption was read in a microplate reader at $A_{595\ nm}$ with $A_{650\ nm}$ as a reference. The data were analyzed using Microsoft Excel's version of the Student *t* test with 2-tailed distribution with sample unequal variance for analysis of variance. The results were presented as % MTT activity where the readings for the control cells were considered as 100%.[23-25]

Osteoblastic Differentiation

Alkaline phosphatase activity. The hTERT-MSCs can be induced to differentiate into osteoblastic, adipogenic, and other lineage under various conditions.[17-19] For the present study, the differentiation protocols used were as described earlier.[26] Briefly, cells were seeded in a 24-well plate at a density of 10 000 cells/well. After 24 hours of attachment period, the normal medium was replaced with ALP-inducing medium (complete DMEM containing 10^{-8} mol/L vitamin D). Cells without HC in normal complete DMEM medium served as undifferentiated controls (UDCs), and cells without HC in ALP medium served as differentiated control (DC). Cells were cultured for 7 days (with media changed on every third day due to the short half-life of vitamin D), after which 3 replicates were used for ALP assay and one used for protein quantification. Alkaline phosphatase activity was assayed by washing the cells twice with phosphate-buffered saline (PBS), adding 250 µL alkaline buffer solution per well, and incubating the cells for 10 minutes at 37°C.; 250 µL of ALP substrate solution (5 mmol/L pNPP diluted in alkaline buffer solution) was added and then plates were incubated for 30 minutes at 37°C. Two aliquots of 200 µL from each well were transferred to the 96-well plate and read in a microplate reader at $A_{415\ nm}$, with $A_{595\ nm}$ as a reference. The commercial ALP was used for making a standard curve and was diluted in the range from 5×10^{-5} to 6.25×10^{-8} U/µL in 200 µL of the ALP substrate solution and incubated in a 96-well plate, simultaneously with the samples. To level out the difference in density from the HC-induced growth arrest, the average ALP activity from each replicate was related to the corresponding cellular protein concentration in the samples.[26]

Mineralized matrix assay. The hTERT-MSCs were seeded in a 24-well plate at a density of 10 000 cells/well. After 24 hours of attachment period, the normal medium was replaced with mineralized matrix (MM) medium (complete DMEM containing 50 µg/mL L-ascorbic acid, 10 mmol/L β-glycerophosphate, and 10^{-8} mol/L vitamin D). Cells without any HC and dexamethasone in MM medium served as negative differentiation controls, and the cells treated with dexamethasone in MM medium served as positive differentiation controls. The cells were cultured for 10 days (media changed on every third day due to the short half-life of vitamin D), after which 3 replicates were used for MM assay and one used for protein quantification. Mineralized matrix assay included Alizarin Red S staining followed by cetylpyridinium chloride (CPC) extraction. Alizarin Red S staining was performed by washing thrice with PBS, fixing with 70% ethanol for 1 hour at −18°C, washing with distilled H_2O, and staining with 500 µL of Alizarin Red S stain, pH 4.2, for 1 hour on a shaker. The cells were washed thoroughly 5 times with distilled H_2O to remove unspecific staining and were incubated in 10% CPC for 1 hour to elute the stain. Two aliquots of 200 µL of eluted stain from each well were transferred to the 96-well plate and read in a microplate reader at $A_{595\ nm}$ and related to an Alizarin Red S standard curve of 0 to10 mmol/L subdilutions in 10% CPC. The values from each well were normalized with the corresponding cellular protein concentration from the sample, and the data were presented as mmol/L Alizarin Red S/µg total protein.[26] For protein quantification, the cells were lysed by a radioimmunoprecipitation assay lysis buffer and the protein was estimated by the DC Protein Assay kit from BIO-RAD according to manufacturer's protocol. All samples were related to a standard curve of bovine serum albumin dilutions in the range of 0 to 2 mg/mL.

Adipogenic Differentiation

The protocol for the induction of adipogenic differentiation in hTERT-MSCs was as described previously.[18,19] Briefly, 30 000 cells/well were seeded in 24-well plates and incubated at normal culturing conditions. Once cells had reached 80% to 90% confluence, complete DMEM was replaced with adipogenic induction medium (AIM: complete DMEM containing 1 µmol/L dexamethasone, 0.2 mmol/L indomethacin, 0.01 mg/mL insulin, and 0.5 mmol/L 3-isobutyl-1-methylxanthin) for 3 days, then followed by 3 days with adipogenic maintenance medium (AMM: complete DMEM containing 0.01 mg/mL insulin) for 3 cycles. Throughout the 3 cycles of adipogenic differentiation induction and maintenance, cells were continuously exposed to different concentrations of HC either in AIM or in AMM conditions. Thus, the cells without HC and without dexamethasone served as negative controls, and cells treated with dexamethasone served as positive controls.[18,19] The formation of intracellular lipid droplets under the influence of HC during adipogenic differentiation was observed under an inverted microscope. The extent of adipogenic differentiation was assessed after Oil red O staining and hematoxylin counterstaining by gently washing twice with PBS and fixing with 4% neutral-buffered formaldehyde at 4°C for 1 hour; 500 µL Oil red O working solution was added and incubated for 15 minutes at room temperature on a shaker. Oil red O-stained lipid droplets appear as red spheres under microscope; further, they tend to form cluster. Counterstaining was done by adding 500-µL hematoxylin and incubated for 1 minute.

Cell Migration/Wound Healing Assay

Cell migration assay, also known as the wound healing assay, was performed as described earlier.[27] Briefly, hTERT-MSCs were seeded at a density of 2×10^5 cells/well in 6-well plates, allowed to attach, grow, and make a near confluent layer in about 24 hours. A scratch was made with custom-made plastic scrapper (2.2 mm width) in the center of confluent layer. Scratched cells in the wells were removed by PBS wash and replaced with fresh complete medium with or without various doses of HC. The wound was allowed to heal for up to 72 hours at normal culturing conditions. One 6-well plate was processed at 0 hour to measure initial wound size. After healing period, the other 6-well plate was processed by fixing cells in 100% ice

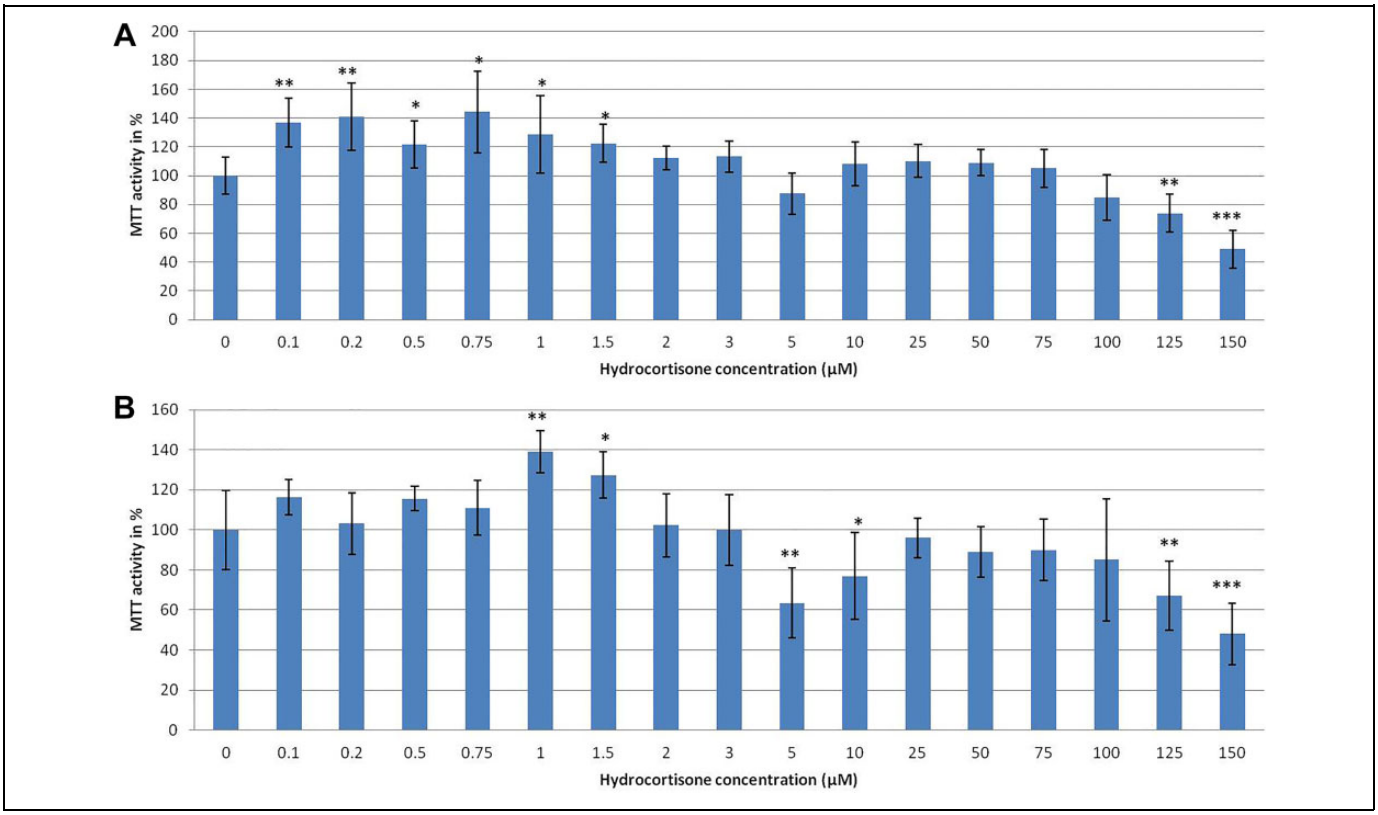

Figure 1. Effect of different concentrations of hydrocortisone (HC) on the metabolic activity of hTERT-MSCs by MTT assay. (A) One-day treatment and (B) 3-day treatment. The results are presented as % MTT activity as compared to the untreated controls taken as 100%. Each bar represents duplicate measures from n = 6 (mean ± standard error of the mean). *$P < .05$; **$P < .01$; ***$P < .001$. hTERT-MSCs indicate telomerase-immortalized human mesenchymal stem cells; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

cold methanol for 30 minutes followed by Giemsa staining. The extent of migration or wound healing was quantified from the scanned plates using ImageJ, image analysis program.[27]

Results and Discussion

Cell Survival, Metabolic Activity, and Morphology

The MTT assay, which is a measure of cell survival and mitochondrial metabolic activity, showed a biphasic dose–response of HC treatment of hTERT-MSCs (Figure 1). Cells were exposed to 16 concentrations of HC between 100 nmol/L and 150 μmol/L either for 1 day (Figure 1A) or for 3 days (Figure 1B). After 1 day of HC exposure, there was a significant increase in the MTT activity of HC-treated cells between the doses of 100 nmol/L and 1.5 μmol/L (Figure 1A). This was followed by a range of HC concentrations (between 2 and 100 μmol/L), where no significant differences between the untreated and HC-treated cells were observed, after which at 125 and 150 μmol/L HC concentrations, a highly significant decline (up to 50% decline) in MTT activities was observed (Figure 1A). A similar biphasic dose–response was seen after 3 days of HC exposure, although the negative/inhibitory effects started to emerge at relatively lower concentrations (5 μmol/L and above) as compared with 1 day exposure (Figure 1B). Based on these MTT results, further studies were done using 1, 10, 50, 100, and 150 μmol/L HC, covering the biphasic dose–response range.

The observed effects of HC on MTT activity could be due to either the changes in the overall metabolic activity of cells or changes in cell numbers owing to altered rates of cell proliferation. Therefore, we determined the number of cells in HC-treated and HC-untreated hTERT-MSCs after 3 and 6 days. Figure 2A shows that although the number of untreated control cells increased by almost 3-fold in 3 days, HC-treated cells at all concentrations increased only by about 1.5 times. A similar situation for cell numbers in HC-treated and HC-untreated cells was observed after 6 days, showing that whereas the number of untreated control cells increased by more than 3.75-fold, the number of HC-treated cells increased to a significantly lesser extent and in a dose-dependent manner (between 2.5- and 1.8-fold) in 6 days (Figure 2B). Thus, any increase or decrease in MTT activity due to HC treatment is uncoupled from its effects on cell survival and proliferation. A comparison of Figures 1 and 2 shows that whereas the effects of HC on the metabolic activity of hTERT-MSCs are biphasic and hormetic, HC treatment slows down the growth and proliferation of cells in general.

The above separation of metabolic alterations and cell proliferation effects of HC is also supported by the morphological

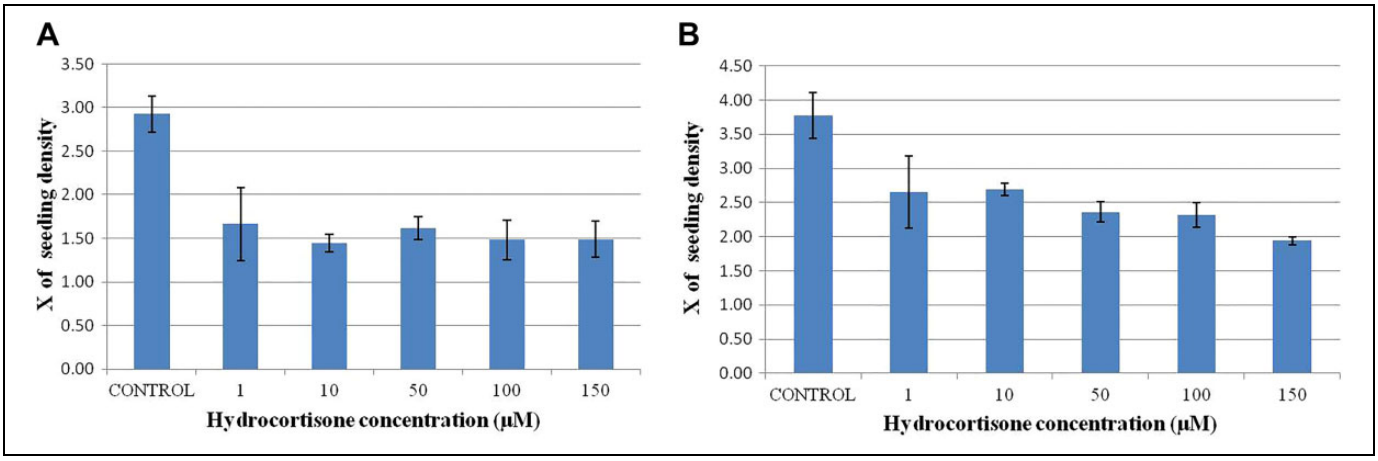

Figure 2. Effect of different concentrations of hydrocortisone (HC) on hTERT-MSCs proliferation. (A) After 3 days of exposure and (B) after 6 days of exposure. Data are presented as times increase (X) in cell number from seeding density. hTERT-MSCs indicate telomerase-immortalized human mesenchymal stem cells.

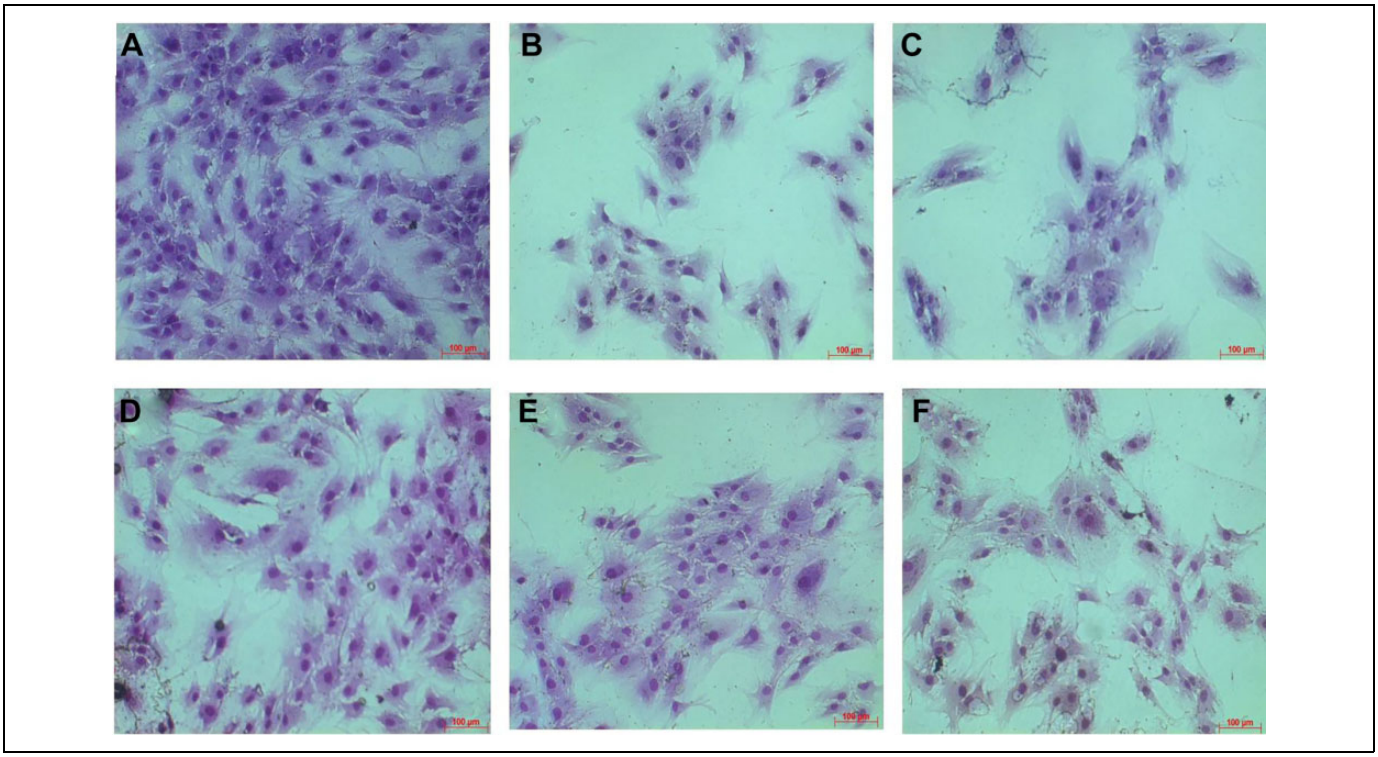

Figure 3. Effect of different concentrations of hydrocortisone (HC) on the cellular morphology of hTERT-MSCs after Giemsa staining. (A) Control; (B) +1 μmol/L; (C) +10 μmol/L; (D) +50 μmol/L; (E) +100 μmol/L; and (F) +150 μmol/L. hTERT-MSCs indicate telomerase-immortalized human mesenchymal stem cells

comparison of HC-treated and HC-untreated cells. Figure 3 shows that hTERT-MSCs treated with various concentrations of HC for 3 days show cytostatic or stress-induction phenotype. For example, although the metabolic activity of 1 μmol/L HC-treated cells is significantly enhanced (Figure 1A and B), these cells appear to be somewhat stressed in terms of becoming more flattened, isolated, and quiescent (Figure 3B). This morphological alteration becomes more significant and apparent at higher doses of HC in a dose-dependent manner (Figure 3C-F). These are the signs of the potentially cytostatic effects of HC on hTERT-MSCs.

Cytostatic effects of HC were further confirmed by the so-called wound healing assay that measures the migration and cell proliferation ability of cells in vitro.[27] Figure 4 shows that HC had a dose-dependent inhibition of cell migration/wound healing: 78.9% healing in 1 μmol/L HC, 69.3% in 10 μmol/L HC, 60.5% in 50 μmol/L HC, 51.7% in 100 μmol/L HC, and 49.2% in 150 μmol/L HC, as compared to untreated controls

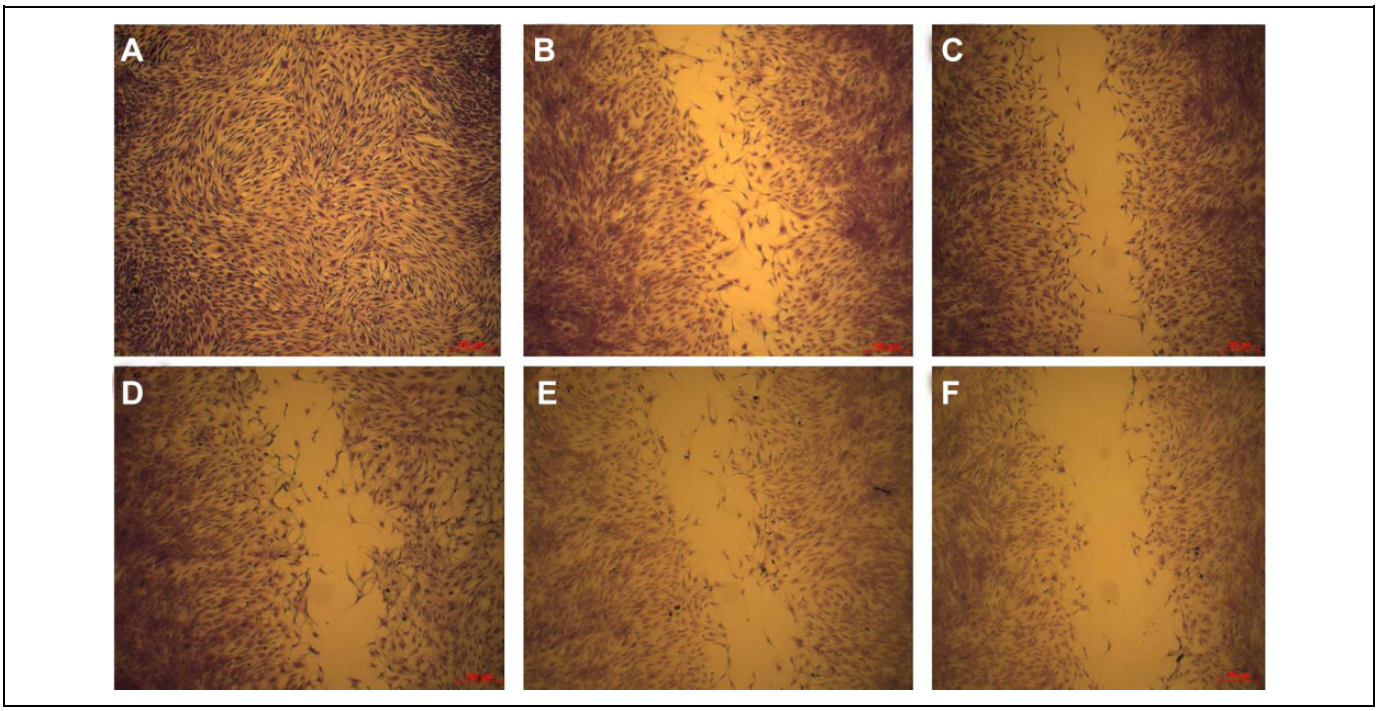

Figure 4. Effect of different concentrations of hydrocortisone (HC) on the migration ability or the so-called wound healing ability of hTERT-MSCs. (A) Control; (B) +1 μmol/L; (C) +10 μmol/L; (D) +50 μmol/L; (E) +100 μmol/L; and (F) +150 μmol/L. hTERT-MSCs indicate telomerase-immortalized human mesenchymal stem cells.

considered as 100%. Similar wound healing reduction effects of HC on cells in vitro have also been reported earlier for CS cells, which otherwise have a longer life span despite being constantly exposed to a high-level HC conditions in vivo.[9,12]

We also investigated whether the cytostatic effects of HC observed above were similar to premature senescence-inducing irreversible effects as reported for other conditions, for example UV irradiation[28] and glyoxal.[29] However, there was no induction of premature or accelerated senescence of HC-treated hTERT-MSCs, as determined by SABG staining[21] (results not shown). The transient nature of HC-induced cytostatic and stressed phenotype effects was also confirmed by removing HC after 3 days and letting the cells recover and proliferate for another 3 days. Figure 5 shows that there was an almost total recovery of cells from the initial negative effects of HC once the stress hormone was removed.

Osteoblastic and Adipogenic Differentiation

The ability of hTERT-MSCs to be converted into several different types of differentiated cells is an important characteristic of their health and functionality.[18,19] Two markers of osteoblastic differentiation (ALP and MM formation) were used after exposure to different culture conditions for the induction of differentiation. Proliferating osteoblasts show greatly increased ALP activity during osteoblastic differentiation assay. The quantified ALP activity in each sample was related to an ALP standard curve and then related to the total cellular protein content of the sample to level out the reduced growth of HC-treated hTERT-MSCs. The data were normalized against UDC (taken as 100%) for comparison and it was found that HC enhanced the ALP activity at both low and high levels. The extent of osteoblastic differentiation under the influence of HC was increased by 118% in 50 nmol/L, 177% in 1 μmol/L, 199% in 100 μmol/L, and 156% in 150 μmol/L, respectively (Figure 6).

The formation of extracellular calcium deposit or MM during osteoblastic differentiation is another confirmatory marker for differentiated osteoblasts.[18,19] The extent of MM formation was determined using Alizarin Red S staining, forms a bright orange-red colored Alizarin red S–calcium complex, which was then quantified after CPC extraction. Figure 7 shows that HC-treated hTERT-MSCs had significantly enhanced (almost 5-fold) MM formation at 1 μmol/L HC level. However, at the highest tested dose (150 μmol/L), this increase in MM formation was lost as compared to the control conditions.

The effect of HC on adipogenic differentiation potential of hTERT-MSCs was also determined by intracellular accumulation of lipid droplets as a marker of differentiation.[18,19] It should be noted that this adipogenic differentiation assay is mainly a qualitative assessment of lipids accumulated and observed under a microscope after Oil Red O staining and hematoxylin counterstaining. Figure 8 shows that HC-treated cells enhanced the adipogenic differentiation at 1 μmol/L HC (Figure 8).

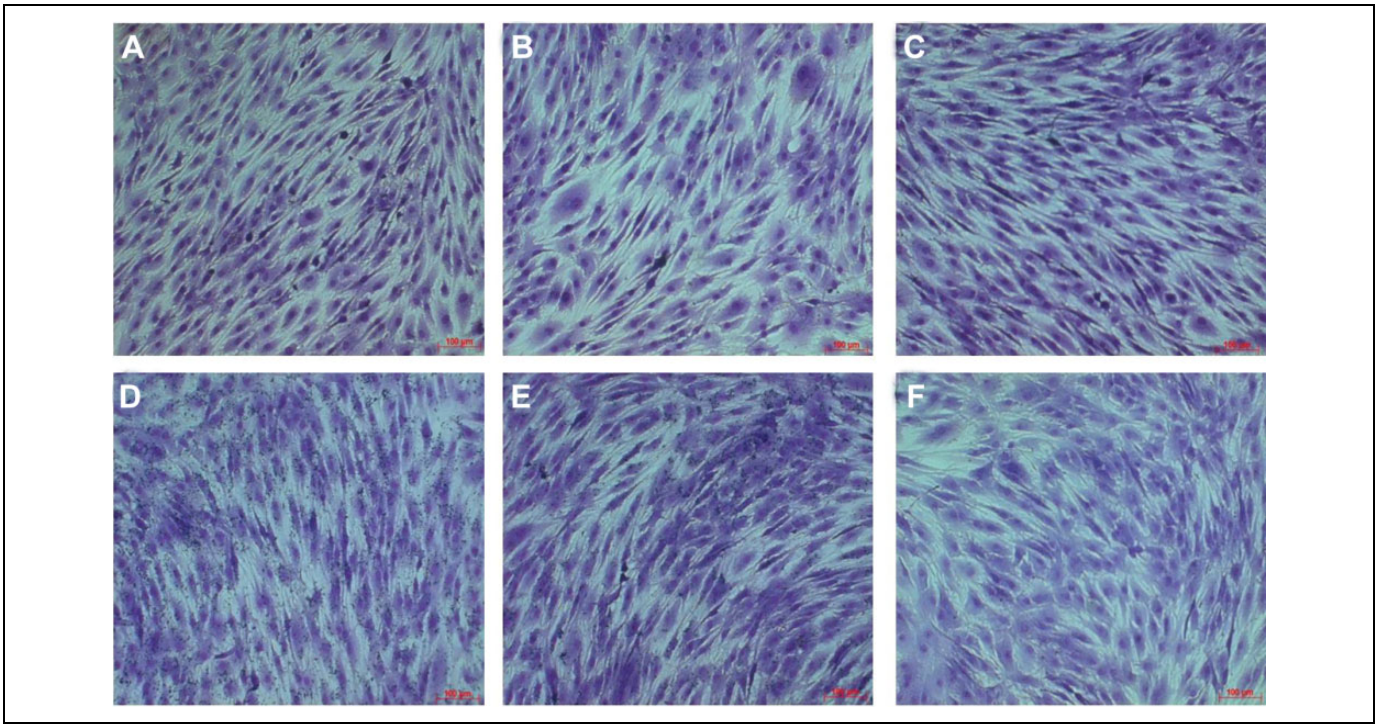

Figure 5. The transient nature of hydrocortisone (HC)-induced cytostatic and stressed phenotype after 3 days of recovery without HC. (A) Control; (B) +1 μmol/L; (C) +10 μmol/L; (D) +50 μmol/L; (E) +100 μmol/L; and (F) +150 μmol/L.

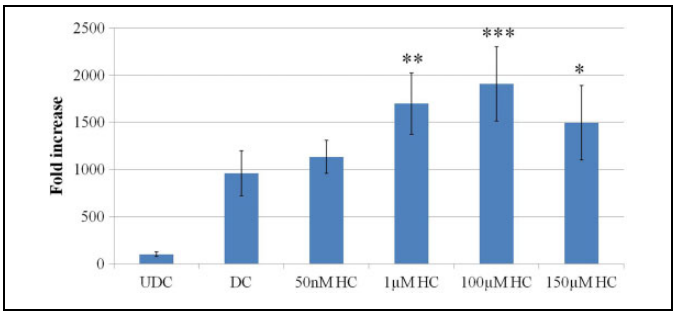

Figure 6. Effect of different concentrations of hydrocortisone (HC) on the alkaline phosphatase (ALP) activity as a marker of osteoblastic differentiation of hTERT-MSCs. Data are presented as "fold increase" from UDC taken as 1. Error bars indicate standard deviation; n = 6; *P < .05; **P < .01; ***P < .001. DC indicates differentiated control; hTERT-MSCs, telomerase-immortalized human mesenchymal stem cells; UDC, undifferentiated control.

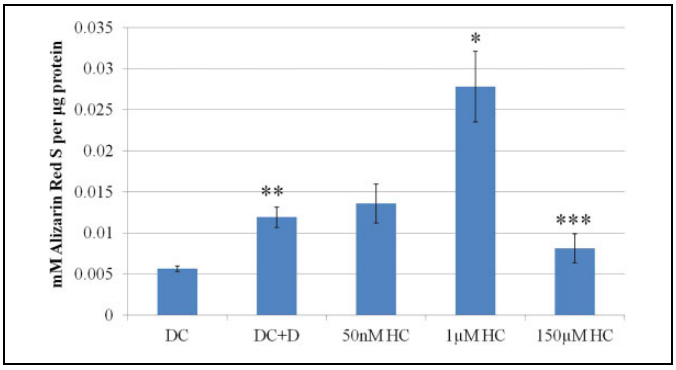

Figure 7. Effect of different concentrations of hydrocortisone (HC) on the extent of mineralized matrix (MM) as a marker of osteoblastic differentiation of hTERT-MSCs. All values were normalized to the corresponding cellular protein content. Error bars indicate standard deviation; n = 6; *P < .05; **P < .01; ***P < .001. DC indicates differentiated controls without vitamin D; DC + D, positive control with vitamin D; hTERT-MSCs, telomerase-immortalized human mesenchymal stem cells.

In conclusion, our findings indicate that hTERT-MSCs exposed to a wide range of HC (100 nmol/L to 150 μmol/L) resulted in a biphasic hormetic dose–response. However, not all measures of cellular characteristics consistently show a biphasic response. For example, the metabolic activity assay clearly shows stimulatory (up to 1 μmol/L) and inhibitory effects (from about 10 μmol/L onward), the cytostatic and differentiation-inducing effects do not show a biphasic dose–response at concentrations between 1 and 100 μmol/L. Although further studies are required to clarify the hormetic and beneficial effects of low-level cortisols at the whole-body level, our present observations provide some support to the potentially hormetic application of HC in maintaining health and longevity. Another aspect of the hormetic effects of low-level HC in inducing osteoblastic differentiation in the presence of vitamin D in vitro is the possibility in vivo of competition of those compounds for the plasma protein transcortin, which can effectively increase the levels of free vitamin D. Both scenarios—differentiation promotion by HC and an increase in free vitamin D in the plasma—may have

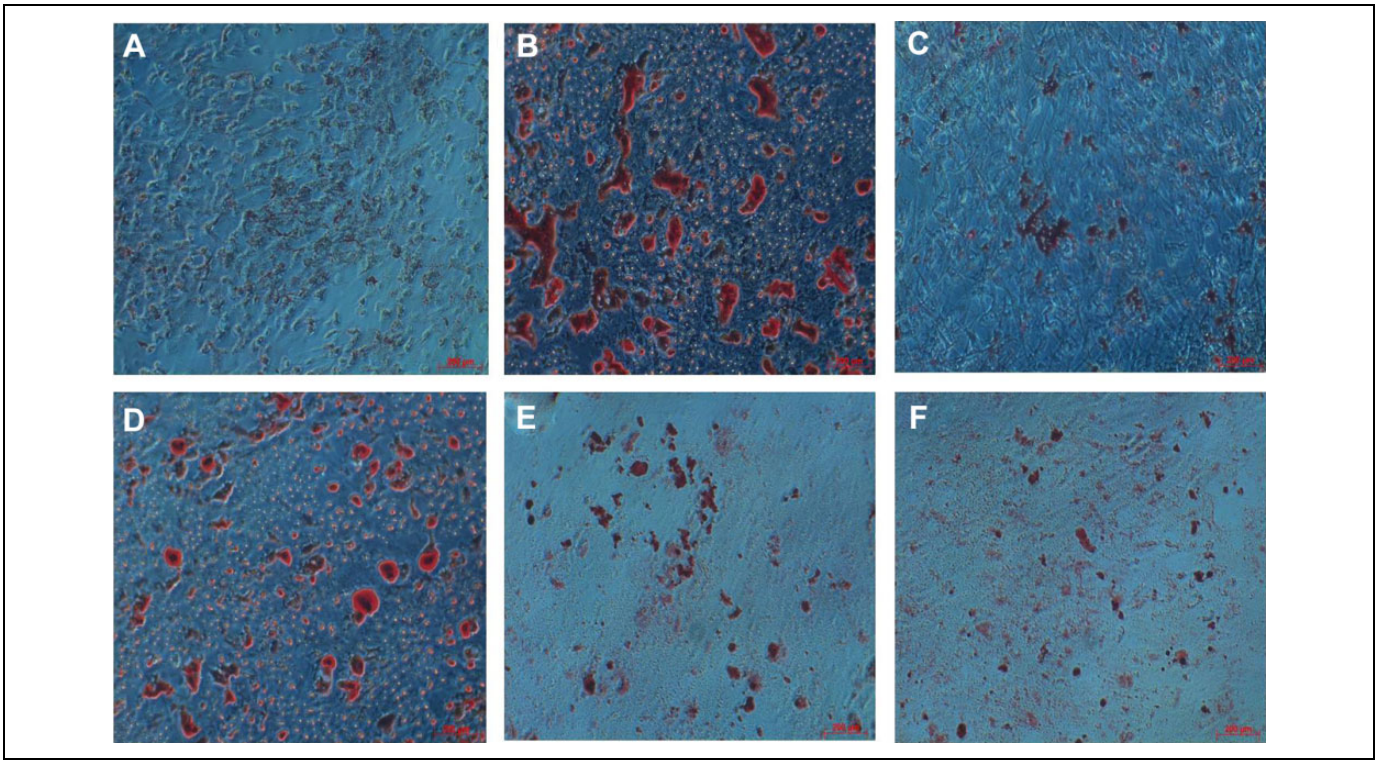

Figure 8. Effect of different concentrations of hydrocortisone (HC) on the extent of adipogenic differentiation of hTERT-MSCs. Lipid droplets appear as red against blue-colored nuclei. (A) Untreated control; (B) +1 μmol/L; (C) +10 μmol/L; (D) +50 μmol/L; (E) +100 μmol/L; and (F) +150 μmol/L. hTERT-MSCs indicate telomerase-immortalized human mesenchymal stem cells.

health-related consequences in vivo,[13] which need further investigations.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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