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ORIGINAL PAPER

Effects of ultra-high dilutions of sodium butyrate on viability and gene expression in HEK 293 cells

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Background: **Several recent studies reported the capability of high diluted homeopathic medicines to modulate gene expression in cell cultures. In line with these studies, we examined whether ultra-high dilutions (30C and 200C) of sodium butyrate (SB) can affect the expression levels of genes involved in acquisition of a senescence-associated secretory phenotype (SASP) in human embryonic kidney (HEK) 293 cells.**
Methods: **Cell viability was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The expression levels of TNF-α, interleukin (IL)-2, IL-4, IL-6 and IL-10 genes were determined by real-time PCR assay.**
Results: **Exposure to both 30C and 200C during 48 h led to a significant decrease of the level of expression of TNF-α gene, while expression of IL-2 gene was increased when exposed to 30C, and expression of IL-10 gene was decreased when exposed to 200C. No changes in expression levels of all genes studied were observed in cells treated with both 30C and 200C remedies of SB during the 24 h.**
Conclusion: **Observed changes in gene expression levels after exposure to 30C and 200C remedies of SB during 48 h suggest that extremely low concentrations of this agent can modulate the transcriptome of HEK 293 cells. These results are in line with findings from other studies confirming the ability of homeopathic remedies to modulate gene expression in cell cultures.** *Homeopathy* (2017) **106**, 32–36.

Keywords: HEK 293 cells; Ultra-high dilutions; Sodium butyrate; Gene expression

Introduction

Since the pioneering works of Samuel Hahnemann, homeopathic medications are widely practiced to treat various pathological conditions. There is however, no known mechanism by which extremely diluted remedies exert their biological effects.[1] The major question is how these preparations can work in the human body. This impedes the recognition of homeopathy as a legitimate health care discipline by scientific community and regulatory agencies. In past years, an intense investigation of putative mechanisms underlying the biological effects of ultra-highly diluted preparations has been initiated. Among other factors, the role of epigenetic processes as a crucial mechanism mediating effects of homeopathic medicine is actively discussed and investigated. Epigenetic changes refer to mitotically and/or meiotically heritable alterations in gene expression that occur without modifications in underlying DNA sequence.[2–5]

The capability of high diluted homeopathic medicines to modulate epigenetic mechanisms of gene expression has been revealed in several recent studies (for review, see, e.g., Bellavite *et al.*[6]). Research evidence of the gene expression-modulating effects has been obtained for a variety of homeopathic remedies. For example, 30C of *Arnica montana* and 30C of *Arsenicum album* have been found to modulate the expression of nucleotide excision repair genes and arsenite-responsive genes in *Escherichia coli*, respectively.[7–9] By studying the effects of Canova (a complex homeopathic preparation used in disorders

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accompanied by depression of the immune system) on cytokine production and gene expression from mice macrophages, a decreased production of IL-2 and IL-4 and a differential expression of 147 genes were observed in the Canova group.[10] Among the genes affected, 45 have been up-regulated and 102 down-regulated relative to the placebo group. Most of these genes are implicated in processes of transcription and translation, enzymatic process, synthesis of receptors and ligands, cell structure and dynamics, cytoprotection and immune response. The altered gene expression profiles were observed in the human prostate epithelial cell lines affected by extremely low concentrations (from 10−6 to 10−17 M) of copper.[11] Increased apoptotic gene p53 expression levels and decreased antiapoptotic gene Bcl2 expression levels were detected in the Dalton's lymphoma ascites tumor cells influenced by potentiated homeopathic drugs such as Carcinosinum 200C and Ruta 200C, respectively.[12] The extreme transcriptional sensitivity was obtained in human SH-SY5Y neurocytes exposed to ultra-low doses of *Gelsemium sempervirens*, a traditional medical plant mainly used in homeopathy as a nervous system relaxant to treat various types of pain and anxiety.[13] Specifically, the expression of 56 genes was shown to be significantly changed, with 49 genes to be down-regulated and 7 to be up-regulated. Most of the down-regulated genes are known to be involved in calcium homeostasis, G-protein coupled receptor signaling, neuropeptide receptors and inflammatory response. In the subsequent study by the same authors, down-regulation of most genes from a panel of human neurotransmitter receptors and regulators involved in neuronal excitatory signaling was revealed in human SH-SY5Y neuroblastoma cells exposed to *G. sempervirens* at 2C and 9C dilutions relative to that of cells treated with control vehicle solutions.[14] In particular, the treated cells have demonstrated a substantial decrease in the expression level of prokineticin receptor 2, whose ligand is a neuropeptide known to be involved in anxiety, nociception and depression-like behavior. In the study by Sunila *et al.*, Carcinosinum 200C was shown to induce significant expression of proapoptotic gene p53 in the mouse fibroblast L929 cell line.[15] In a very recent whole-genome transcriptomic analysis, Bigagli *et al.* revealed the significant effects of a wide range of *Apis mellifica* dilutions (3C, 5C, 7C, 9C, 12C, 15C, and 30C) on gene expression profiles in a non-neoplastic adult human epithelial prostate cell line, RWPE-1.[16]

In line with these studies, we investigated the effects of ultra-high dilutions of sodium butyrate (SB), a sodium salt of butyric acid ($CH_3CH_2CH_2COONa$), in immortalized human embryonic kidney (HEK) 293 cell line, widely used as an *in vitro* model system. SB was selected as promising for the screening because it is a powerful inhibitor of histone deacetylase activity, i.e., belongs to a novel class of drugs targeting epigenetic pathways.[17] In a number of studies, it has been found that relatively low concentrations of this compound can cause a wide variety of effects in cultured mammalian cells including inhibition of cell proliferation and induction of differentiation.[18] Moreover, since SB may induce apoptosis in cancer cells,[19] it was proposed as a promising therapeutic agent for cancer treatment.[20] In our previous studies, it has been also found that SB in concentrations ranging from 10 to 40 mM can substantially extend life span in *in vivo* model such as *Drosophila melanogaster*.[21,22] In similar studies by other authors, elevated levels of expression of *hsp22*, *hsp26*, and *hsp70* genes were found in SB treated flies,[23–27] suggesting that alterations in histone acetylation and, thereafter, the expression of chaperone genes, may contribute to the life-extending effects of SB. In our present study, we determined the effects of homeopathic dilutions (30C and 200C) of SB to examine whether such ultra-high dilutions can affect the expression levels of genes known to be involved in the acquisition of a senescence-associated secretory phenotype (SASP), in HEK 293 cells.

Materials and methods

Cell culture and exposure conditions

The HEK 293 cell line has been cultured in Greiner plastic culture flasks in DMEM medium (Farmak, Ukraine) enriched by 5% of fetal bovine serum (Sigma−Aldrich, USA) supplemented with antibiotics (streptomycin, 100 mg/ml; penicillin, 100 mU/ml) and maintained at 37°C with 5% CO_2 in a humidified atmosphere (90% humidity). The culture medium was replaced every three days. Cells were counted in duplicate in a Thoma counting chamber after staining with Turk blue reagent.

Cells were propagated in T-75 culture flacks (Nest biotechnology, China) at 370C in 5% CO_2 atmosphere, until 60% of confluence and then exposed to the 30C and 200C SB during 24 h or 48 h. 15 drops (∼3 ml) of each of studies remedies as well as placebo were diluted in 10 ml sterile water for injections (Yria-pharm, Ukraine) dynamized by vigorous shaking. Control cells were exposed to the same volume of placebo (33% ethanol) solution. Three replicate experiments have been carried out under identical conditions.

Table 1 The sequences of the primers used for real-time PCR

Gene	*Forward primer (5′ → 3′)*	*Reverse primer (5′ → 3′)*
TNF-α	CCGAGGCAGTCAGATCATCTT	AGCTGCCCCTCAGCTTGA
IL-2	AAGAATCCCAAACTAACCAGG	TCTAGACATGAAGATGTTTCAGTTCTC
IL-4	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
IL-10	ATGCCCCAAGCTGAGAACCAAGACCCA	TCTCAAGGGGCTGGGTCAGCTATCCCA
β-actin	TAATGTCACGCACGATTTCCC	TAATGTCACGCACGATTTCCC

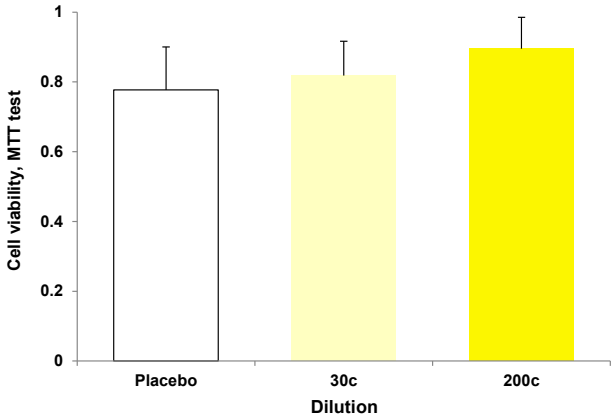

Figure 1 MTT assay of HEK 293 cells treated with 30C and 200C remedies of sodium butyrate. Bars represent means with standard deviation (n = 8).

Cell proliferation assay

Cell viability was evaluated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. This assay quantifies viable cells by measuring the conversion of the yellow MTT to purple formazan by mitochondrial succinate dehydrogenase, according to the manufacturer's instructions (Sigma–Aldrich, USA). The absorbance of the supernatant was measured at 590 nm using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek). Based on the absorbance of the cell samples, cell viability was measured. Cell viability was expressed as the amount of dye reduction in treated cells relative to that of untreated control cells.

Real-time PCR

HEK 293 cells were propagated after reactivation of cryogenates until the fourth culture passage and then used for

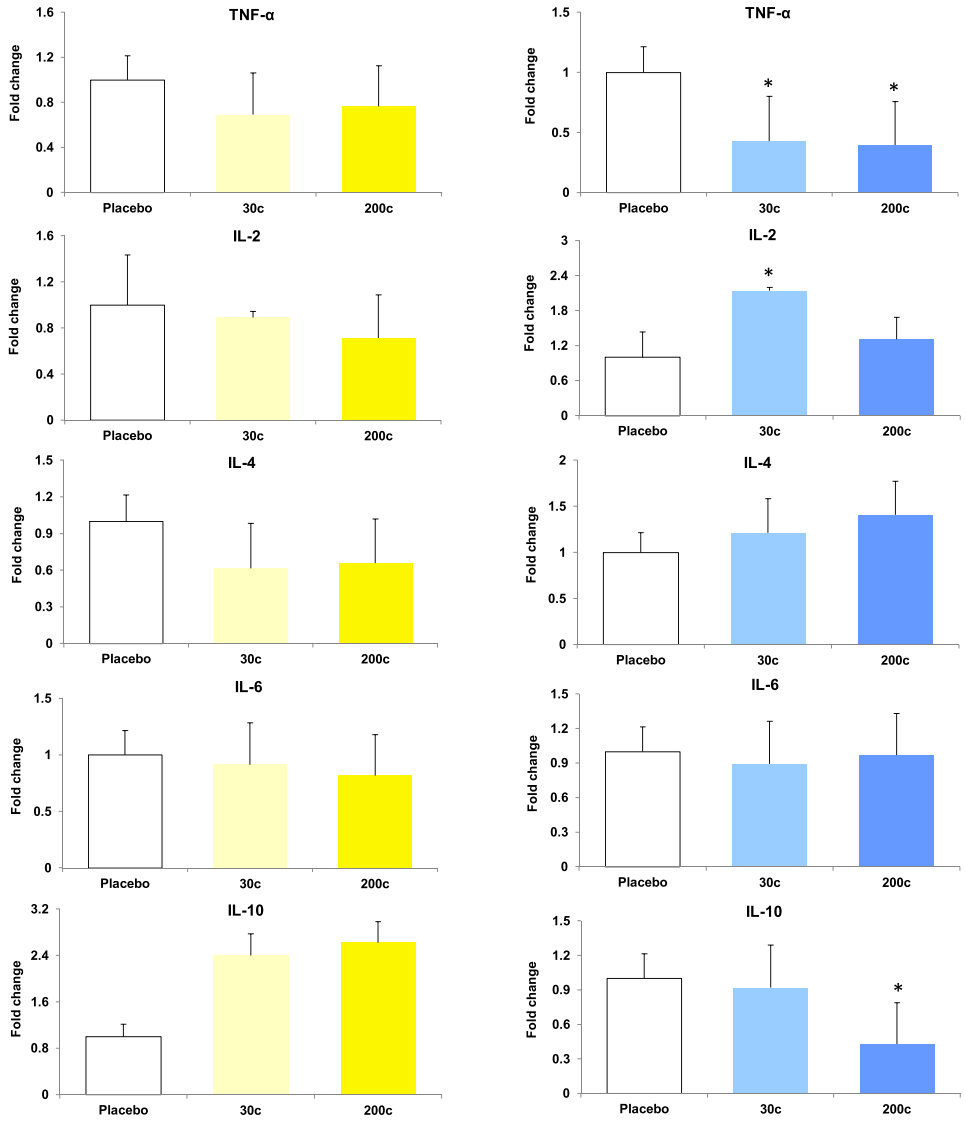

Figure 2 Quantitative real time PCR of genes of interest exposed to 30C and 200C of SB, and to placebo during 24 h (left panels) or 48 h (right panels). The transcript levels of genes are expressed as fold changes normalized with β-actin gene. The bars represent the standard deviations and asterisks indicate statistical significance at *p < 0.01.

the gene expression assay. Total RNA was isolated using RNeasy kit (Qiagen, Gaithersburg, MD) and any contaminating DNA removed using TURBO DNA-free (Ambion, Austin, TX), both according to the manufacturer's recommendations. Reverse transcription of 250 ng total RNA was performed in a total volume of 20 μl using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. One microliter of cDNA was PCR amplified in 25 μl reactions containing primers at 250 nM and SYBR Green Supermix (Bio-Rad). PCR amplification was performed for 36 cycles 1 min at 94°C, 1 min at 59°C, 40 s at 72°C.

Five cytokine genes [interleukin (IL)-2, IL-4, IL-6, IL-10 and TNF-α] were selected for analysis. β-actin gene was used as an internal control for normalization of RNA quantity. The sequences of the primers used for the real-time PCR are presented in Table 1. Negative controls, including cDNA reactions without reverse transcriptase or RNA, and PCR mixtures lacking cDNA were included in each PCR. Following amplification, specificity of the reaction was confirmed by melt curve analysis. Relative quantitation was determined using the comparative CT method with data normalized to β-actin and calibrated to the average ΔCT of untreated controls.

Statistical analysis

Treatment effects were assessed by one-way ANOVA using the treatment as factor, followed by Tukey's HSD test to compare the means. The data are presented as the mean $\pm$ standard deviation of triplicate samples. Differences were regarded as significant at p-value less than 0.01.

Results

Treatments with homeopathic remedies used did not lead to cell death or proliferation inhibition estimated by MTT-assay (Figure 1).

Exposure to both 30C and 200C remedies during 48 h resulted in some cases to significantly altered levels of gene expression. Specifically, these treatments affected expression of the TNF-α gene [ANOVA, $F(2,6) = 15.99$, $p = 0.004$], IL-2 gene [ANOVA, $F(2,6) = 60.62$, $p = 0.0001$] and IL-10 gene [ANOVA, $F(2,6) = 33.28$, $p = 0.0006$]. *Post hoc* pairwise comparisons demonstrated that exposure to both 30C and 200C led to a significant decrease of the level of expression of TNF-α gene, while expression of IL-2 gene was increased when exposed to 30C, and expression of IL-10 gene was decreased when exposed to 200C (Figure 2). At the same time, no changes in expression levels of all genes studied were observed in HEK 293 cells treated with these remedies during 24 h. It can be assumed that a much longer exposure is required to induce such effects.

Discussion

Observed changes in gene expression levels after exposure to 30C and 200C remedies of SB during 48 h suggest that SB-based homeopathic medicines can modulate the transcriptome of HEK 293 cells. These results are in line with findings from other studies confirming the ability of various homeopathic remedies to modulate gene expression.[7–16] For our analysis, we selected five cytokine genes known to play a role in acquisition of SASP, which involves the production of factors that reinforce the senescence arrest, alter the microenvironment, and trigger immune surveillance of the cells thereby significantly contributing to aging process both *in vitro* and *in vivo*.[27] The SASP occurs because the intracellular damage accompanying aging-associated processes, such as epigenetic alterations, genomic instability, telomere attrition and loss of proteostasis, cause loss of cell functionality. A permanent proliferative cell-cycle arrest (commonly referred to as cellular senescence) occurs in damaged cells to prevent their propagation in the organism.[28] Under the normal conditions, senescent cells recruit immune cells through cytokine secretion thereby providing their removal from tissues. Throughout the aging process, however, senescent cells tend to accumulate in the organism, and they may unfavorably affect their microenvironment.[29] Such cells secrete many pro-inflammatory chemokines, cytokines, and extracellular matrix proteases, which collectively constitute the SASP, thereby contributing to systemic dysfunction and chronic disease.[30]

Our research findings and those of others show that homeopathic medicines could both turn on or turn off particular sets of relevant genes, triggering a cascade of gene expression changes, thereby affecting important biological processes including the formation of SASP. Further studies are however needed to test this hypothesis.

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