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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