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Effect of ultrasounds on neurons and microglia: Cell viability and automatic analysis of cell morphology

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ABSTRACT

Ultrasounds, besides their well-established medical imaging role, influence the homeostasis of complex anatomical systems including the physiology of neurons and glia and the permeability of the blood brain barrier. In this study, neurons and microglial cells were treated with ultrasounds (commonly used in diagnostics) and differences in cell proliferation and morphology were evaluated in comparison to control, untreated cells. Cell proliferation was evaluated by standard viability assessment, while the quantitative analysis of cell morphology, usually performed by edge and line detection algorithms, required the development of a new special algorithm. In fact, traditional software methodologies do not provide the appropriate tools for morphological analysis of neurons and microglial cells, typically characterized by a roughly triangular body and numerous elongations of different lengths resulting in a complex neuron–microglia network. This new method, based on a modified Hough Transform algorithm using a matching operator instead of the common gradient filter, enabled the automatic identification of cell elongations and branches, the extraction of related information, and the comparison of the data between control and treated neurons, as well as microglial cells.

Results, based on the development of the new algorithm, showed that in ultrasound-treated cells, the number of elongations, as well as their maximum and mean lengths, increased significantly in comparison to control, untreated cells.

These results were consistent with the standard microscopic evaluation. Furthermore, a significant correlation between cell morphology and proliferation suggested that ultrasounds induced cell differentiation affecting cell morphology, as well as the ability of neurons and microglial cells to form complex networks. Our results suggest the possibility of using ultrasounds, currently utilized in diagnostics, to reconstitute neuronal and microglial circuits that are often altered in neurodegenerative and neurodevelopmental disorders.

1. Introduction

The incidence of neurodegenerative and neurodevelopmental disorders is on the rise worldwide for a variety of reasons ranging from population ageing to exposure to toxicants [1,2]. The enormous and increasing disease burden of these disorders raises the urgency to understand the underlying causes, as well as to develop efficient treatments.

Several approaches and numerous drugs exist to protect or restore the normal function of central nervous system (CNS), and to increase viability of neurons, glial cells and complex neuronal networks impaired in neurodegenerative and neurodevelopmental disorders. Unfortunately, their therapeutic efficacy is often limited because of side effects [3].

Recent data have shown that ultrasonography, and in particular focused ultrasounds, positively affect the blood–brain barrier [4]. These observations led us to hypothesize that the non-focused ultrasounds, currently used in the field of medical diagnostics, may elicit positive effects on cell differentiation, as well as on the morphology and connectivity of those cells of the CNS impaired in neurodegenerative and neurodevelopmental diseases. Neuronal and glial connectivity, as well as the complex neuronal network in the CNS, represent the disorders main areas of concern in these conditions [5]. Within the scientific literature, only a few studies

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examine the effects of ultrasounds on the functional connectivity and neuron–glia networks in the CNS [6]. Furthermore, due to the complexity of the interaction between neurons and glial cells, as well as the highly particular shape of these cells, we required more reliable and efficient *in vitro* methods to objectively analyse and assess neuronal and glial connectivity.

With the aim of evaluating the effects of ultrasounds on cell morphology and on the interactions between neurons and microglial cells, we developed an automated image analysis method focused on the detection and analysis of cellular elongations. We validated the results of this new automated methodology by comparison to standard visual morphological evaluation. Furthermore, we assessed the effects of a common ultrasound treatment on the proliferation of neurons and microglial cells *in vitro*. These results correlated with standard and automated morphological evaluations.

Unlike other cell types, neurons and microglial cells possess a roughly triangular body and numerous branched elongations of different lengths creating very complex networks. For these reasons, the standard quantitative image analysis softwares for cell morphology based on edge and line detection algorithms are inaccurate and unreliable for the morphological study of the neurons, glial cells, and their interconnections.

The earliest attempts to develop automated detection and analysis of cells in cyto-histological specimens date back a few decades [7]. Various different approaches exist ranging from relatively simple threshold techniques, to more sophisticated model-based methods using *a priori* information on cell shape features to obtain a separation of cell clusters and a cell/nucleus segmentation in microscopic images [8]. Some of those methods used are Markov random fields [9], stepwise merging rules and gradient vector flow snake [10], and combined fuzzy clustering with multiphase vector level sets [11]. These various methodologies exemplify the diversity used to tackle this complex problem [12]. Recently, the automated image analysis of cells has received increased attention especially in biomedical research and clinical pathology. While qualitative and semi-quantitative visual inspections of morphological features are considered the standard approach in diagnostics and research, cyto-histological analyses for the evaluation of the stage of a disease or its response to treatment now require accurate and valid standardized quantitative assessment [13].

In the case of cells of the nervous system, cellular image analysis should focus on the identification and morphology of the cellular outline (more than that of the nuclei). Importantly, the cytoplasmic elongations of neurons and glial cells forming complex circuits have a deep functional relevance since their loss, or decrease, is involved in numerous neuropathologies [14,15].

The approach of our automated image analysis method presented and discussed in this paper, employs a modified Hough Transform algorithm incorporating a matching operator instead of the common gradient filter. This new algorithm efficiently enables the automatic identification and evaluation of a cell's branched elongations. This then permitted the analysis of the related information, including the comparison of the data between controls and ultrasound-treated samples.

2. Results

2.1. Cell proliferation

After the treatment with 12 MHz ultrasounds for 3 min, proliferative activity of neurons was significantly decreased ($p < 0.05$) in comparison to control untreated cells (Fig. 1A).

Similar results were obtained for the microglial cell line BV-2. As shown in Fig. 1B, when cells were treated for 3 min with 12 MHz ultrasounds, their proliferative activity significantly decreased ($p < 0.05$) in comparison to control untreated cells.

2.2. Standard morphological analysis

We performed a contrast phase microscopic evaluation on cells immediately after the treatment with ultrasounds. As shown in Fig. 2, control untreated neurons SH-SY5Y, were characterized by a typical triangular cell body, spindle-like shape, and several

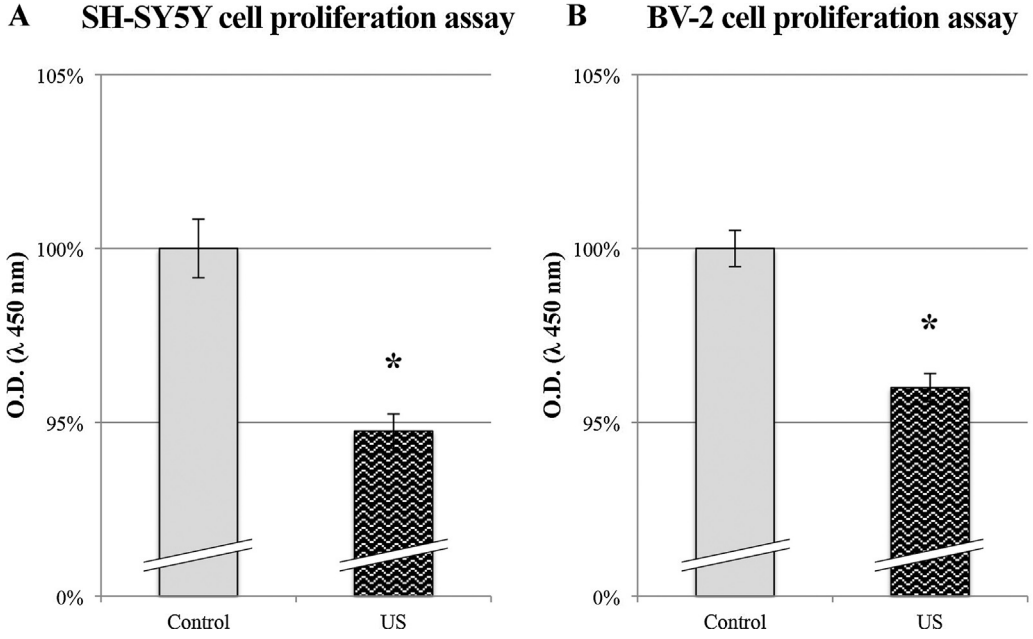

Fig. 1. *WST-8 cell proliferation assay.* (A) Proliferative activity of neurons SH-SY5Y is significantly decreased in comparison to control untreated cells after the treatment with ultrasounds. (B) Proliferative activity of microglia BV-2 is significantly decreased in comparison to control, untreated cells after the treatment with ultrasounds. In this figure, to clearly visualize the differences between the proliferative activity of control and ultrasound treated cells, the proliferative activity of controls has been considered as 100 and the proliferative activity of ultrasound treated samples has been normalized on the control. Results are expressed as mean value ± S.E.M. for two replicates (* $p < 0.05$ *vs.* control); each experiment was performed three times. O.D.: optical density. US: ultrasounds.