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Review

Biological effects and applications of bulk and surface acoustic waves on in vitro cultured mammal cells: new insights

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Abstract: Medical imaging has relied on ultrasounds (US) as an exploratory method for decades now. Nonetheless, in cell biology, the numerous US applications remain mainly in the research and development phase. In this review, we report the main effects on human or mammal cells of US induced by bulk or surface acoustic waves (SAW). At low frequencies, bulk US can lead to cell death. Under specific intensities and exposure times however, the cell proliferation and migration can be enhanced through cytoskeleton fluidization (a reorganization of the actin filaments and microtubules). Cavitation phenomena, frequencies of resonance close to those of the biological compounds, and mechanical transfers of energy from the acoustic pressure could explain those biological outcomes. At higher frequencies, no cavitation is observed. However, US of high frequency stimulate ionic channels and increase the cell permeability and the transfection potency. Surface acoustic waves are more and more exploited in microfluidics especially for precise cell manipulations and cell sorting. With applications in diagnosis, infection, cancer treatment, or wound healing, the US have remarkable potential. More mechanotransduction studies would beneficiate the field though, to grasp the distinct roles of temperature rise, acoustic streaming, mechanical and electrical stimuli.

Keywords: ultrasounds; surface acoustic waves; mammal cells ; cytotoxicity; proliferation ; migration ; cell permeability; cell sorting ; wound healing

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1. Introduction

Ultrasounds (US) are widely used in the medical field, and increasingly in the wider area of biotechnologies. The most famous application is medical imaging, using frequencies from 1 to 10 MHz, and an intensity lower than 720 mW.cm^{-2} [1]. In this context, the US show remarkably no or negligible toxicity towards the biological tissues. Low intensity pulsed ultrasounds have been shown to enhance enhanced tissue repair or bone regeneration [2,3]. Other medical applications of US were researched. At low frequency, a phenomenon called cavitation, can create transient pores in the cell membranes and locally increase the delivery of therapeutic drugs through translocation [4]. At higher frequencies, transfection can be achieved without cavitation, allowing gene or protein delivery with great applications potential in oncology [5,6]. US can be triggered by the resonance of a whole bulk material (as shown in Erreur ! Source du renvoi introuvable. A and B), or the resonance of the extreme surface of an elastic material (Erreur ! Source du renvoi introuvable. C). These surface acoustic waves (SAW) are due to a piezo-electric system stimulated by an interdigital transducer (IDT). They allow microfluidic manipulations of very small volumes to single Erreur ! Signet non défini.cells, and could enhance wound healing [7,8]. Studies of US physical and molecular mechanisms of action are a rising field across physical and biological sciences.

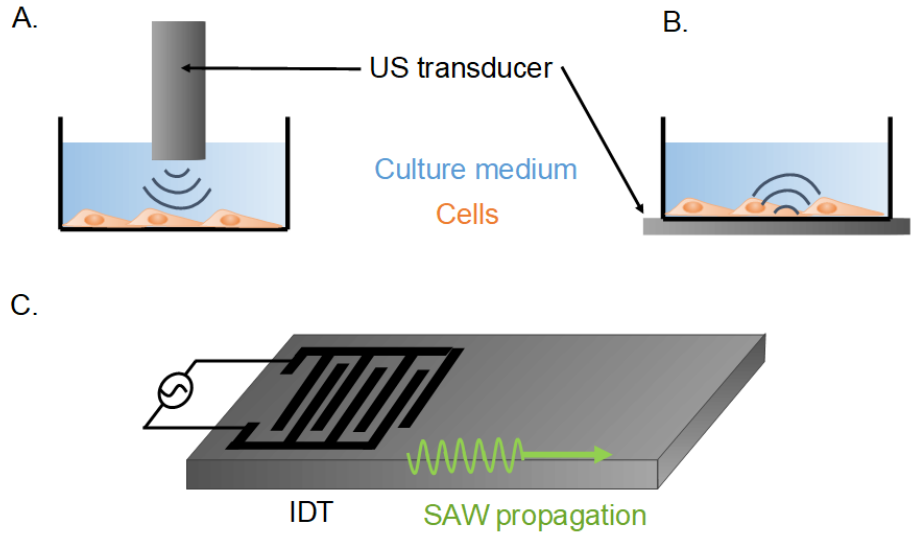

Figure 1. Schematic view of cell-stimulation systems of low or high intensity ultrasound stimulation. A: Cells stimulated mainly by the shear flow induced by an US transducer immersed in the culture well. B: Cells stimulated mainly by the mechanical vibrations of the culture well US stimulated by the US transducer under it. C: Piezo-electric system with an interdigital transducer (IDT) inducing surface acoustic waves (SAW).

In this article, we review all studies on the action of US with frequencies over 1 MHz on human or mammal cells. Studies are split as a function of the US frequency: first from 1 to 10 MHz ; and over 10 MHz. The latter coincides with more recent works. This review is then centered on SAW, excluding standing SAW for concision and to avoid redundancies with the latest articles on the subject [9–11]. Biological outcomes are questioned, as well as the physical phenomena that trigger them: cavitation, mechanical stimulation, or acoustic streaming.

Before starting to review the literature on this field, let us define some terms linked to US stimulation characterization. On the Erreur ! Source du renvoi introuvable., the main parameters are drawn. The wave frequency (in Hz) is reciprocal to the period (in s). Stimulations often are in pulse mode, with a duty cycle defined as the ratio stimulation time (ON time) on total time (ON time + OFF time). The duty cycle is equivalent to the pulse period, thus reciprocal to the pulse repetition frequency. The dose, expressed in $J.cm^{-2}$ or $W.s.cm^{-2}$, is defined as the product of the intensity, expressed in $W.cm^{-2,}$ and the exposure time, expressed in s.

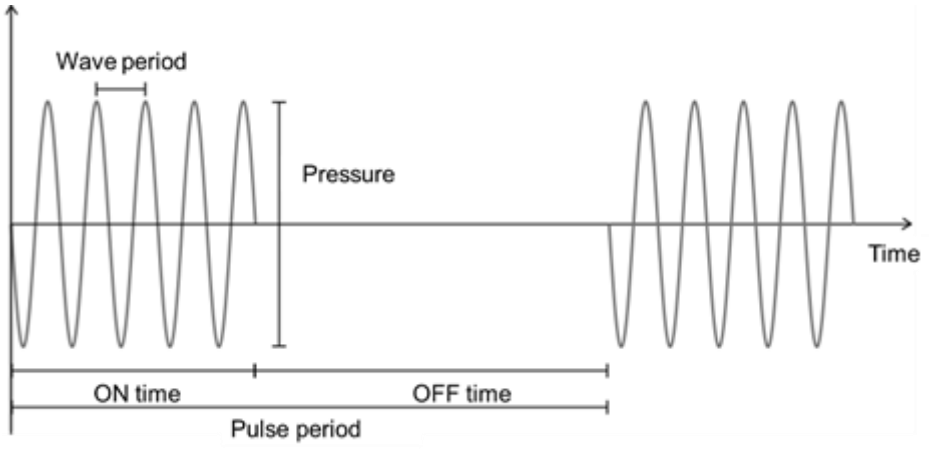

Figure 2. Graphical representation of the parameters defining ultrasonic stimulations.

2. Systematic review method

2.1. Research design

Objective: To review the scientific literature on the impacts of ultrasounds generated by acoustic waves on mammal cells, on in vitro models.

Inclusion criteria: peer reviewed articles or book chapters, referenced in scientific databases, in English, containing at least 2 of the keywords, no criteria of publication date

Exclusion criteria: study focusing on standing surface acoustic waves, pour scientific quality, or a study that does not provide enough parameters for the comprehension and comparisons of its results.

2.2. Selection and extraction of the studies

Keywords: surface acoustic waves, acoustic waves, ultrasounds, cells*, bio*, (effects OR impacts).

Data sources: We identified suitable studies by searching electronic databases and scanning reference lists of articles. We searched Web of Science, Google scholar.

Selection of the studies in two times: A first selection was carried out by two peoples (LO, DB), independently, for a primary view of the problematics and a focus on low frequency ultrasounds (< 10 MHz). Two authors (AF, DB) independently assessed the eligibility of studies for second validation, more focused on high frequency ultrasounds (10 to 1000 MHz) and ultrasounds induced by surface acoustics waves, as well as the inclusion of newer studies on the three topics. Any disagreements were settled by consensus. Other authors could suggest a particular study, if not selected yet, the study was checked for compliance with inclusion or exclusion criteria and selected accordingly.

2.3. Analysis of the studies

Literature reviews were read and included as part of the discussion. Three summary tables were built reporting the main parameters and results of the studies: one for the low frequency ultrasounds, one for the high frequency ultrasounds, and the latter for the ultrasounds induced by surface acoustic waves. For the last part, the electrical power to the IDT had sometimes to be extrapolated from the voltage (root mean square peak, or peak to peak) across the electrode, making the hypothesis that the electrode impedance is at 50 Ω. There is no information about the electrical impedance of IDTs, but given the expertise in the field, the error made on the electrical power with this assumption should be small enough to consider the order of magnitude. Indeed, classically, some SAW devices have an electrical impedance matching circuit in order to increase the energy transfer between the energy source and the SAW device. If there is no impedance matching, the standing wave ratio (SWR) of the IDT never exceeds 1.5, which means that 80% of the incident electrical energy is transmitted to the IDT and therefore 20% is reflected. A SWR of 1.2 to 1.3 is closer to reality for standard bi-directional electrodes, which translates into nearly 90% of the incident energy being available at the IDT. The error made by making this assumption (50 Ω) will therefore be, at most, only 10 to 20%, which is acceptable and allows us to have a good order of magnitude to compare the works between them.

3. Ultrasounds at low frequencies (< 10 MHz)

Biological effects of US at low frequencies have been extensively studied as they are extensively used for medical investigations [2,12–15]. The following sections are devoted to the study of their potential impacts on human and mammal cells as a function of the US frequencies and exposure time. The Erreur ! Source du renvoi introuvable. recapitulates those findings and was elaborated to ease the reading of this review.

Table 1. Summary table of the impacts on mammal cells of US stimulation at frequencies under 10 MHz. (↗: increase in, ↘: decrease in, N.A.: not available).

Reference	Frequency (MHz)	Intensity or pressure	Duty cycle (%)	Pulse time (min)	Dose ($J.cm^{-2}$)	Cells	Temperature control	Biological effects	Hypothesis
[16]	0.045, 1	10-400 $mW.cm^{-2}$	25	5	7.5-75	Primary fibroblasts Primary osteoblasts Primary monocytes	Rise ≤ 1.8°C	↗ proliferation ↗ collagen synthesis	N.A.
[17]	1	100-400 $mW.cm^{-2}$	10	1	0.6-2.4	Human monocytes (U-937) T lymphoblasts (Molt-4) Lymphocytes (Jurkat) Leukemia cell line (HL 60)	Rise ≤ 1°C	↗ DNA double strand breaks if I > 200 $mW.cm^{-2}$	Free radicals formation, due to cavitation.
[18]	1	300 $mW.cm^{-2}$	50	0,5 - 15	4.5-135	Human adenocarcinma epithelial cells (HeLa)	None	↗ membrane permeabilization ↗ intracellular transport	N.A.
[19]	1.8	7 $mW.mL^{-1}$	65	0,33	91 $J.mL^{-1}$	Human leukemia bone marrow cells (K562, KG1a) HL-60, human B cell precursor leukemia cells (Nalm-6)	None	↗ apoptosis Mild necrosis Virulent leukemic cells more sensitive	Oxygen singlet formation, due to cavitation.
[20]	1.48	0.045 MPa	15-70	5-30	N.A.	Rat pheochromocytoma adrenal medulla cells (PC-12)	None	↗ proliferation	N.A.
[21]	1	250 $mW.cm^{-2}$	20	30	90	Mouse osteoblasts (MC3T3-E1)	Pre-heated water tank	↗ proliferation ↗ migration	N.A.
[22]	1	1000-2000 $mW.cm^{-2}$	20	0,5	6-12	Human aortic smooth muscle cells (HASM)	Rise ≤ 1°C	Reversible fluidization for I = 1000 $mW.cm^{-2}$ Damages to the actin filaments for I = 2 $W.cm^{-2}$	Fluidization due to the compression wave causing a local cell deformation
[23]	1	800-1000 $mW.cm^{-2}$	50	0,25	6-7.5	Human oral squamous carcinoma cells (HSC-2) U-937	None	↘ HSC-2 viability with microbubbles. No effect on U-937. No effect without microbubble.	N.A.
[24]	0.5, 1, 3.5, 5	1600-2000 $mW.cm^{-2}$	10-100	30	288-3600	Endothelial cells	Measured temperature "excluded the possibility that thermal effects may cause	↗ proliferation ↗ cytoskeleton disorganization ↗ tissue repair.	direct mechanical action

							changes in the cultured cells"		
[25]	0.5, 1, 3, 5	250-1000 mW.cm^{-2}	20	5	15-60	Mouse myoblasts (C2C12)	Room temperature (28°C) water tank	↗ proliferation ↗ differentiation	Mechanical constraints
[26]	0.8, 1.5	150, 250 kPa	100	0,17-0,5	N.A.	C2C12	Rise ≤ 1°C	Induce cytoskeleton fluidization ↗ cell mortality	Cell deformation with acoustic pressure
[27]	0.51, 0.994, 4.36	N.A.	N.A.	N.A.	3, 25, 50	Human cardiac microvascular endothelial cells (hcMEC) Madin-Dabry Canine Kidney cells (MDCK) Mouse neuroblastoma cells (Neuro2A) Human colon cancer cells (HT29)	Perfused water tank at 37°C	↗ proliferation at low I Not anymore at high intensity	N.A.
[28]	0.51, 4.36	N.A.	N.A.	N.A.	3, 25	Neural stem cells	Perfused water tank at 37°C	↗ proliferation no increase in neurogenesis or gliogenesis	N.A.
[29]	1	70-300 mW.cm^{-2}	100	30	126-540	HeLa Human fetal lung fibroblasts (MCR-5) Human breast cancer cells (MCF-7)	Rise ≤ 1°C	↗ mitotic abnormalities as a function of I disassembly of focal adhesions and microtubules.	N.A.

3.1. Adverse effects on cells

US can trigger apoptosis and low level of necrosis, as shown on leukemic cells exposed at low frequencies of US generated by a ceramic disk (1.8 MHz frequency, 7 mW.mL^{-1} intensity, exposure from 1 to 18 h) [19]. The hypothesis, proposed by Lagneaux et al., is that the apoptosis is triggered by the presence of the $^1O^2$ oxygen singlet, the instable and highly reactive state of dioxygen, due to the sonoluminescence caused by a cavitation phenomenon [19].

Genotoxicity appears to be another effect of this inertial cavitation phenomenon, either linked to oxidative stress or to the mechanical constraints of the cavitation alone. DNA double strand breaks caused by these forces were evidenced in leukemic cells exposed to US at 1 MHz frequency, with a 10% duty cycle, namely a 100 Hz pulsed wave, an intensity higher than 200 mW.cm^{-2}, and an acoustic pressure higher than 0.105 MPa [17]. Udroiu et al., showed nevertheless that US can affect the genome integrity even at intensities below the cavitation threshold [29]. Transient mitotic anomalies were observed after a 30 min US stimulation at 1 MHz, and an intensity of either 70, 140, or 300 mW.cm^{-2} defined by the authors as respectively below, around, or over the cavitation threshold. This genotoxic effect was retrieved in different cell types: HeLa human cervical cancer cells, MCR-5 human pulmonary fibroblasts, and MCF-7 human breast cancer cells.

Adverse outcomes from US exposure may however depend on the cell type. Lagneaux et al. exposed that cancerous cells seemed to be more sensitive to US induced necrosis than non-cancerous cells [19]. Other researchers have studied how to induce selective cell death. Narihira et al. have studied the effects of US in the presence or absence of

Cetuximab (an anticancer drug) coated albumin microbubbles on oral squamous carcinoma cells (HSC-2 cells) and tumor monocytes (U-937) [23]. The cells were exposed to US of 1 MHz, with a 10 Hz repetition pulse frequency, and a duty cycle of 50%. Intensities of 0.8, 0.9, and 1 W.cm^{-2} were delivered for 15 s, which corresponds of 150, 160, and 170 kPa pressures. Whatever the intensity, the viability decreased in a dose-dependent manner in HSC-2 cells only.

3.2. Proliferation, cytoskeleton rearrangement and transfection

When the parameters are properly calibrated, US can enhance cell proliferation and migration. Studies bore interest on wound healing, or bone regeneration. Using acoustic intensities from 30 to 1000 W.cm-2 and frequencies between 1 to 3 MHz, US could positively affect the differentiation and protein synthesis of osteoblasts, osteoclasts, chondrocytes and mesenchymal stem cells [2]. At 1 MHz, 250 W.cm^{-2} with a duty cycle of 20%, the proliferation rate of murine osteoblasts increased by 20% [21]. The speed of the scratch-wound healing increased with US stimulation even when the proliferation was blocked with mitomycin C, hence a conclusion on an increase migration as well as the proliferation by US. The parameters for a maximal proliferation seem, once again, to depend on the cell types. On murine myoblasts (C2C12), the most efficient parameters to increase proliferation were 3 MHz and 1W.cm^{-2} (20% duty cycle, negligible medium heating), but 1 MHz, 500 mW.cm^{-2} for differentiation [25]. On rat pheochromocytoma cells (PC-12) however, if a 138 to 186% increase in proliferation was seen, no significant difference between the stimulation parameters was observed. The used frequency was 1.48 MHz, the maximal pressure 45 kPa, 15, 30, 50, 70% duty cycle, 5, 10, 20, 30 min stimulation 3 times a day for 3 days [20]. In another study, at 1 MHz, 0.1 to 1 W.cm^{-2}, a significant rise in primary osteoblasts and fibroblasts proliferation was also observed (47% or 37% at 0.7 or 1 W.cm^{-2} for osteoblasts, and 34% or 52% for fibroblasts) [16]. Interestingly, the collagen synthesis rose as well at 0.1 to 0.7 W.cm^{-2} or 0.1 to 0.4 W.cm^{-2} for fibroblasts and osteoblasts respectively.

The US impacts on cytoskeleton and proliferation were questioned in several recent studies. Raz et al. hypothesized that cell sonication induces transient alterations leading to cytoskeleton reorganization, cell proliferation and migration (Erreur ! Source du renvoi introuvable., top) [24]. Those effects were linked to mechanical energy transfer to the cells, increasing as a function of the US frequency until reaching a plateau. A 60% increase of cells proliferation was evidenced in bovine endothelial cells following 15 to 30 min 1.2 W.cm^{-2} US stimulation with a frequency of either 0.5, 1, 3.5, or 5 MHz, and a duty cycle of 50 and 100%. At 15 min a difference in cell proliferation was seen between the duty cycle of 50 and 100%, but this disappeared at 30 min. The study moreover underlined morphological changes in actin fibers, disassembly of their focal-adhesions and microtubules (Erreur ! Source du renvoi introuvable., bottom). Initial states were recovered after 24 h, supporting thus the authors' hypothesis. Focal-adhesion are constituted mainly by integrin, which has been shown to be activated by low-intensity pulsed ultrasounds [3]. These effects on the cytoskeleton organization and cell proliferation appear to be a function of the cell types. Schuster et al. have indeed demonstrated that for an equivalent US dose, no impact on actin and focal-adhesions but an increase in proliferation was observed for a human cardiac microvascular endothelial cell line (hcMEC) [27]. Moreover, the proliferation rate of a Madin–Darby Canine kidney epithelial cell line (MDCK) increased with the US energy until 25 W.s.cm^{-2} then started to decrease. For a mouse neuroblastoma line (Neuro2A cells) or a human colon adenocarcinoma cell line (HT29), the proliferation rate increased only at high energy (600 W.s.cm-2) and dropped at low energy. In addition to the proliferation rate, electronic microscopy showed an increased number of cells presenting plasma membrane blebs, which might be a sign of apoptosis (Erreur ! Source du renvoi introuvable. top). Using a similar protocol, a second study showed an increase in neural stem cell proliferation, but no impact on neurogenesis and gliogenesis [28].

Other studies sought to better understand this phenomenon of cytoskeleton disorganization, and showed its "fluidization" under US stimulation. The fluidization is a phenomenon where soft materials go from solid to a fluid-like state when submitted to a shear stress [30]. In cell biology, the so-called cytoskeleton fluidization indicates a reorganization of the actin fibers and microtubules, leading to deformations of the plasma membrane. In Mizrahi et al. study, the cytoskeleton of human Airway Smooth Muscle cells (HASM) showed such fluidization under US stimulation at 1 MHz. Following an exposure of US at 1W.cm^{-2}, 20% duty cycle (to minimize the temperature rise to under 1°C), the effects were transient, and a repolymerization of the actin filaments was observed in 200 s. At 2 W.cm^{-2} however, the effects were irreversible and US lead to the cell death. The fluidization could be due to the compression waves that generate a local deformation of the cell [22]. Samandari et al. developed a simulation model, and compared it to their experimental outcomes. Their standard linear solid viscoelastic model showed that the cell deformation increases with the pressure. These deformations might depolymerize the actin filaments and activate signaling pathways sensitive to mechanotransduction. The deformations are more important when the cell is spread out and close to the substrate. In their experiment, C2C12 cells were stimulated with US at a 0.8 or 1.7 MHz frequency, generating a pressure of 150 or 250 kPa, for 10 to 30 s. The temperature rise stayed below 1°C. No cavitation was observed. Cell death increased with pressure and frequency, even though it remained below 15%. Microscopic observations showed, as expected, a rearrangement of the actin cytoskeleton and blebs formation [26]. No studies have yet underlined the effects of US on the cytoskeleton intermediate filaments: vimentin, keratin, lamin, desmin, etc.

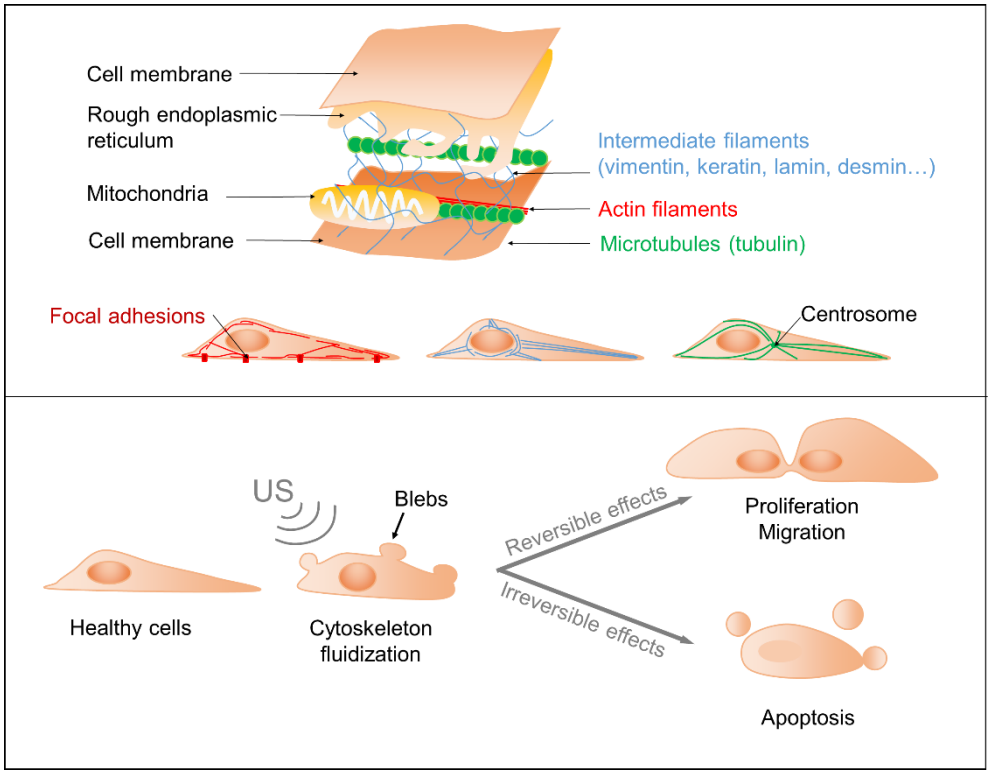

Figure 3. Cytoskeleton and main impacts from US exposure. Top: Schematic outcomes of US on cellular cytoskeleton, proliferation and migration. Bottom: Schematic visualization of the cytoskeleton components. Focal-adhesions are integrin-containing multi-proteins structures binding actin filaments to the extracellular substrate.

As a consequence of the effects on the cell cytoskeleton, US can temporarily disrupt the cell membrane. This property was used for transfection: the controlled introduction of

exogenous genetic material such as gene or proteins into a cell (Erreur ! Source du renvoi introuvable.) [4]. As a proof of concept, plasmid DNA was transfected to HeLa cells exposed to US at 1 MHz, 300 mW.cm^{-2}, 50% duty cycle, and 5 Hz pulsation frequency [18].

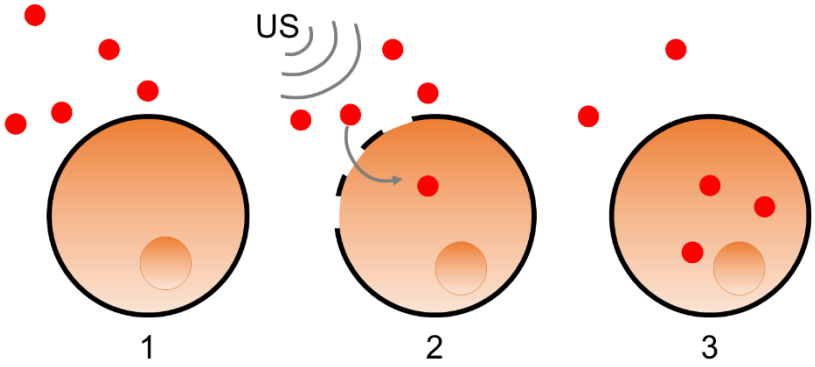

Figure 4. Schematic description of gene or protein transfection. The elements to transfer are in the extracellular medium (1). The cell membrane is disrupted by US (2). The cell membrane closes again after integration of the transfected elements.

3.3. Towards an understanding of the physical mechanisms of action

Several teams tried to formulate hypotheses and propose models to explain the physical phenomena at play in the biological effects of US. The resonance and shear stress forces could provoke the disjunction between molecular complexes or conformation changes of biomacromolecules. The hypothesis presented by Johns suggests indeed that the absorption of US energy by the enzymes could lead to their activation. The link between an enzyme and its inhibitor may be broken, or the enzyme may adopt an active conformation on its own. In both cases, the biochemical reactions that the enzyme catalyze will be boosted [31]. Other biomacromolecules could be affected, such as the lipids forming the cell membrane. A study, published in 2011, suggested that the US mechanical energy impacts the hydrogen bonds between the two phospholipid layers of the plasma membrane and transforms it by contracting and expanding the intramembrane space [32]. These constraints could explain the cytoskeleton reorganization, and eventual potential membrane disruption, with irreversible impacts at high frequencies. The cavitation and ensued microbubbles might act as amplifiers of the phospholipids' reorganization.

Cumulative effects of the stress impacted by the resonance on the organelles could also lead to a fatigue phenomenon, which explains the observed cellular damages. Or and Kimmel [33] developed a model to understand the impacts of US on cell membranes , without thermal and cavitation effects. Frequencies varying from 0.001 to 100 MHz were applied onto objects of 100 nm, 1 µm and 5 µm radius. Four rheological models were tested: viscous fluid, elastic solid, and Voigt and Maxwell viscoelastic constructs. It was shown that the resonance frequency, the frequency for an intracellular vibration of maximal amplitude, was radius dependent. 100 nm radius objects, of similar size as cell organelles, resonated at 1 MHz, a current frequency for medical applications. Miller et al. confirmed such findings for chondrocytes (12 µm radius) with a maximal deformation whatever the pressure of US considering a resonance frequency of 5.2 MHz. At other frequencies, the deformation increased with the pressure but to a lesser extent [34].

The impacts of US depend on physical parameters such as resonance frequency and acoustic pressure, but also on biological parameters such as the cell size, adherence, and type. The frequency, pressure or dose units are not sufficient to comprehend the US effects on cells. A review from 2007 [35] stated that mW.cm^{-2} the most used intensity unit, even if simple and easy to apprehend, does not explain the acoustic field characterization at the

studied area. The acoustic shear was rarely taken into consideration in the reviewed studies. Nevertheless, these studies opened up paths to better understand the physical phenomena at play for low frequencies, or as seen in the next paragraph, for frequencies higher than 10 MHz.

4. Ultrasounds at high frequencies (10-1000 MHz)

At high frequencies, the cavitation phenomenon is not observed; moreover, the beamwidth becomes narrower allowing a more precise cell stimulation. Technologies using US at frequencies higher than 10 MHz were recently developed, such as single cell imaging [36,37]. We will focus here in direct impact on cell behavior with applications in the cellular or medical sciences as summarized in Erreur ! Source du renvoi introuvable..

Table 2. Summary table of the impacts on mammal cells of bulk US stimulation at frequencies from 10 to 1000 MHz. (↗ : increase in, ↘: decrease in, N.A.: not available, **: extrapolation based on the hypothesis that the electrode impedance is at 50 Ω*).

Reference	Frequency (MHz)	Voltage, intensity or electrical power	Duty cycle (%)	Pulse time (s)	Dose (J.cm^{-2})	Cells (adherent)	Temperature control	Biological effects	Hypothesis
[38]	15 + LED	47.9, 82.15, 128.11 mW.cm^{-2}	100	1800 (daily)	126 000 – 230 600	Human cervix carcinoma cells (HeLa)	None	↘ proliferation	N.A.
[39]	200	16, 32, 47 V *110, 230, 330 mW**	2.5	10	N.A.	Human breast cells (MCF-12F) Human breast cancer cells (MDA-MB-435)	Thermally controlled chamber	↗ cell permeability higher in non-cancerous cells	N.A.
[40]	200-1000	4, 8, 16, 32 V *30, 60, 110, 230 mW**	0.0025-1	0.3-150	N.A.	Highly invasive human breast cancer cells (MDA-MB-231) Weakly invasive human breast cancer cells (MCF-7, SKBR3, and BT-474)	None	↗ Ca^{2+} influx as a function of invasiveness	N.A.
[41]	193	1.8-3.6 MPa	0.1, 0.25, 0.5, 0.75, 1	0.5	N.A.	Endothelial cells (HUVEC)	Thermally controlled chamber	↗ Ca^{2+} influx	N.A.
[42]	43	50,000, 90,000 mW.cm^{-2} *3.2, 5.7 mW focused on 1 cell*	100	0.7	35, 63	Chinese hamster ovary cells (CHO) expressing rat $Na_v1.2$ or mouse piezo 1 channels Human embryonic kidney cells (HEK) expressing mouse piezo 1 channels	Estimated rise of 0,8°C	Stimulation of the $Na_v1.2$ and piezo channels	US through acoustic radiation and shear stimulate the piezo channel Thermal heating stimulates the $Na_v1.2$ channel
[5]	50	0.43-1.97 MPa	33	3.3	N.A.	Human breast cells (MCF-10A) MDA-MB-231 MCF-7	Rise ≤ 0.5°C	↗ Ca^{2+} influx, as a function of invasiveness	US stimulate the piezo channel

[6]	150, 215	22-43 V *160-300 mW**	100	0.016, 0.023	N.A.	HeLa	None	Size and amount of transfected elements depend on the voltage, duration, frequency and number of US pulsation. No impact on viability	N.A.
[43]	150, 215	22 V *160 mW**	0.0036	0.5-1.5	N.A.	HeLa	None	Genomic transfection facilitated by US	N.A.

4.1. Activation of ion channels, applications in oncology and neurostimulation.

At first, studies have shown that the permeability enhancement by US at high frequencies, or high frequency microbeam stimulation (HFUMS), seemed to depend on the invasive nature of the cells. Hwang et al. showed that US at 200 MHz increased cell permeability more significantly for human breast non-cancerous cells compared to cancer cells, as evidenced by Rhodamine B reflux [39]. Higher voltage induced higher impact on permeability. Following studies concluded that HFUMS can enhance the cell permeability through the activation of specific ion channels [40,41]. Ion channels are membrane proteins allowing the transport of a specific ion or a family of ions down the electrochemical gradient (see Figure 5). They are ubiquitous, crucial for the physiology of excitable cells, especially neurons, and their activity is modified in cancerous cells. A significant difference in Ca^{2+} influx was indeed observed following exposure to US of 193 MHz on human breast cancer or non-cancerous cells, and US of 200 MHz on endothelial cells (HUVEC) [40,41]. Likewise, another study found no impact of US at 50 MHz on human breast non-cancerous cells, but an increase in Ca^{2+} influx, as a function of the invasiveness of the human breast cancer cells [5]. Another class of cationic channel, the transient receptor potential (TRP) channel, could also play a part, but no significant activation by US was observed. All those studies relied on fluorescence index as a sensor of Ca^{2+} concentration changes. Transcriptomic analysis of the genes involved in the piezo channel or the TRP channel would be welcomed. The detection of the difference in cell response towards HFUMS could allow the specific distinction between non-cancerous and highly invasive cancer cells. Moreover, this kind of stimulation by US showed no impact on cell viability, displaying optimal parameters for potential applications of HFUMS as biosensors [5]. In addition to being a tool for diagnosis, HFUMS could help tumor treatment. Daily exposure to a 30 min period of HFUMS in combination with light-emitting diodes (LED) induced significant decrease in the proliferation of human cervix carcinoma cells (HeLa) [38,44]. This effect was shown for a frequency of 15 and 100 MHz, and a range of intensity higher than 100 $W.cm^{-2}$. Similar proliferation drops were found at 100 MHz with US only (no LED) [45]. The authors supposed this could open a new path for cancer treatment.

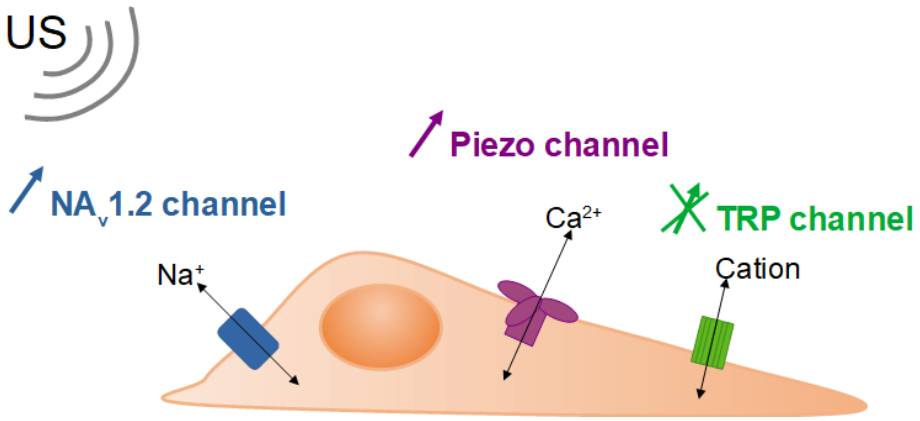

Figure 5. Schematic description of cellular ionic channels: $Na_v1.2$ and piezo channels were shown to be activated by SAW, while no significant impact on TRP (transient receptor potential) channel was observed.

Prieto et al. [42] conducted further work on the activation of ionic channels by HFUMS in hope of developing applications in neurostimulation, and treatments against mental and neurological disorders (Prieto et al., 2018). The study used Chinese hamster ovary cells (CHO) modified to express mouse piezo 1 channel, or rat $Na_v1.2$ channel: a type of sodium channel, or as well as human embryonic kidney cells (HEK) modified to express mouse piezo 1 channels. The cells were exposed for 0.7 s to US at a frequency of 43 MHz, and an intensity of 50 or 90 $W.cm^{-2}$. This work confirmed the activation of the piezo channel by the US, more specifically by the acoustic radiation pressure and streaming. The $Na_v1.2$ channel was also activated by the stimulation, albeit only due to thermal heating. Heating can indeed activate or speed the kinetic of ion channels. It has to be noted that the temperature rise at play here was only of 0.8°C. Thus even a small difference in environmental temperature due to US could impact the cell response.

4.2. Increase in permeability and transfection

Using the US effects on the membrane permeability through channel activation, the HFUMS can additionally be used to transfect small molecules, DNA plasmids and RNA messengers. At high frequencies, no microbubble is needed. HFUMS enables thus controlled and local intracellular delivery of chosen molecules. Such US-transfection system was developed by Yoon et al. at 150 and 215 MHz and tested on HeLa cells. Size and amount of transfected fluorescent dextran molecules depended on the frequency, the number of electric pulses, the peak-to-peak voltage (V_{pp}), and pulse duration (t_p). The study optimized the parameters for a maximal transfection of 3 kDa dextran molecules, without any significant impact of cell viability at short (6 h) and long (40 h) term. The optimized parameters were: V_{pp} = 22 V and t_p = 30 µs, or V_{pp} = 43 V and t_p = 10 µs [6]. A year later, the same team used this method to successfully transfer CRISPR-Cas9 systems and succeed in reprogramming the genome of HeLa cells [43]. HFUMS-transfection was thus confirmed as an efficient technique for efficient genome editing.

5. Ultrasounds induced by surface acoustic waves

The previous sections focused on bulk acoustic waves, where the whole transducer resonates to produce ultrasounds in the environmental medium. Here, we will study the impacts of surface acoustic waves (SAW), where only the extreme surface of an elastic material resonates. The SAW are also called Rayleigh waves, in reference to the name of the first scientist to describe them in 1885. The generation of SAW requires the conversion of electrical to mechanical energy. A voltage is applied to a metallic interdigitated transducer (IDT) on the surface of a piezoelectric substrate, generally a lithium niobate ($LiNbO_3$) chip, on the surface of which acoustic waves propagate longitudinally (Erreur !

Source du renvoi introuvable. bottom). These acoustic waves can propagate to other adjacent media as cell culture plate and culture medium, in which they create an acoustic streaming. Cells can thus be directly stimulated by mechanic waves or by shear flow. Due to the small size of these microsystems and relatively low cost, SAW have a wide range of cell manipulation applications, refining and completing those of bulk acoustic waves (Erreur ! Source du renvoi introuvable.) [46,47].

Table 3. Summary table of the impacts on mammal cells of SAW stimulation. (↗: increase in, ↘: decrease in, N.A.: not available, AFM: atomic force microscopy, IDT: interdigital transitor, PDMS: polydimethyl siloxane, **: extrapolation based on the hypothesis that the electrode impedance is at 50 Ω*).

Reference	Frequency (MHz)	Intensity or electrical power	Duty cycle (%)	Time	Shear flow	Device	Cells	Temperature control	Biological effects	Hypothesis
[48]	10	65 - 250 mW	N.A.	N.A.	N.A.	Slanted IDT, $LiNbO_3$ chip	Human red blood cells (RBC) RBC infected by the malarial parasite *Plasmodium falciparum*	None	Enrichment, separation of the cells depending on their pathological state	Cell density impacts their displacement with the shear flow
[7]	77-164	80-1000 $mW.cm^{-2}$ up to 13.6 mW	100 or 0.00077	5 min - 27 h	N.A.	$LiNbO_3$ chip covered with a SiO_2 layer (= substrate), PDMS well	Madine–Darby canine kidney (MDCK-II) Human osteosarcoma sarcoma osteogenic (SaOs-2) Human embryonic kidney (T-REx-293)	Estimated rise of 2.4°C	Wound healing ↗ cell migration ↗ cell proliferation	Direct mechanical stimulation > flow field, or electrical field
[49]	101-204	380 mW	100	seconds	N.A.	4 IDT, $LiNbO_3$ chip	Human lymphocytes RBC infected by the malarial parasite *Plasmodium falciparum*	Thermally controlled chamber	Patterning of spatially isolated individual cells in an acoustic field defined in 2D	N.A.
[50]	48.8	467 mW	2.5	48 h	Shear stress 120-280 $mN.m^{-2}$ Shear velocity 600 ± 250 $\mu m.s^{-1}$	$LiNbO_3$ chip, titanium substrate, PDMS well	Human monocytes (U-937)	Rise ≤ 0.5°C	↗ cell proliferation (+ 36%)	Shear stress linked to SAW has a more positive impact than stirring
[51]	14	Up to18 V, 59.3 $mW.cm^{-2}$ and 0.23 μW for a single cell (400 μm^2) *order of magnitude up to 100 mW**	100	4-8 h	Velocity up to 56 $\mu m.s^{-1}$, shear stress 3.8 mPa	$LiNbO_3$ chip, glycerol as a coupling liquid with the PDMS cell culture chamber	Mouse embryonic fibroblasts (NIH-3T3)	Feedback loop to maintain the temperature of the medium flow	Cell migration first enhanced, then suppressed as the intensity rose No reduction in cell viability	Cell orientation alignment along the propagating wave, high traction forces activated the Rho

									Thicker actin bundles	signaling pathway
[52]	160	631 mW	100	60 min	Shear rate distribution 1750-6900 s^{-1}	Gold IDT, $LiNbO_3$ chip, a cylindrical PDMS chamber on top filled with culture medium, cells attached to a titanium implant on top	SaOs-2	Temperature maintained at 37°C, no precision	Correlation between shear flow and cell detachment from an implant	Cell density plays a key role
[53]	19.35	325 - 575 mW	100	10 s	Velocity 0-9 $mm.s^{-1}$	$LiNbO_3$ chip, titanium layer, aluminum substrate,	none	/	↗ penetration rate into a porous scaffold	N.A.
[54]	161 - 171	31.6 mW	N.A.	> 330 µs per pulse	N.A.	Gold and titan $LiNbO_3$ chip, covered with glass, PDMS microchannel device	Mouse melanoma cells (B16F10)	None.	Sorting rate of 3000 $cells.s^{-1}$ depending on their fluorescence (Calcein-AM)	N.A.
[55]	196.7	1 mW 10-20 kPa	100	3-10 min	N.A.	Quartz (SiO_2) chip, cells suspended in glycerin, SU-8 microprobe	Chondrosarcoma (JJ012) Breast cancer cells (MDA-MB-231, SKBR3, MCF7)	None	US velocity measurement for single cell analysis 10^6 sensitivity in elasticity than AFM	Cell elastic moduli is a possible biomarker for aggressiveness or metastatic potential
[56]	132	55-500 mW	100	100 s	Velocity 0.42-1.80 $m.s^{-1}$ Shear stress 0.01-0.045 Pa	Concentric gold IDT, $LiNbO_3$ chip	Untreated, and non-infected human RBC Glutaraldehyde-treated RBC RBC infected by the malarial parasite	None	Cell detachment behavior was different according to the RBC state of infection.	Specific mechanotransduction might be a biomarker
[57]	159	2-4 mW	100	48 h	N.A.	$LiNbO_3$ chip, SiO_2 substrate, PDMS well	SaOs-2	Rise ≤ 0.32°C	↗ wound healing as a function of US intensity no significant necrosis no preferred direction for migration/proliferation	Unclear if the effect is due to mechanical stimulation, electrical or a combination of both

[58]	N.A.	316 - 501 mW	100	0-60 min	Shear flow 2 Pa	$LiNbO_3$ chip, titanium substrate	SaOs-2	Thermally controlled chamber	No significant impact on cell adhesion, when T ≤ 37°C	Decrease in cell adhesion is due to increase in temperature or decrease in pH
[8]	38.74	125.6 mW	80	2 h	N.A.	Two circular IDT (and two straight IDT for SSAW), $LiNbO_3$ chip, covered with Al, and PDMS channels	Human glioma cell lines (U87) Rat RBC	None	Cell sorting depending on their virulence	Sorting of particles is dependent on their size

5.1. Controlling cell detachment

A time-dependent detachment of human cells from their growing substrate can be observed when exposed to SAW. Likewise Stamp et al. applied power of 300 to 500 mW applied to a $LiNbO_3$ chip, inducing SAW and US (no information was given about their frequency and intensity) that detached adherent human osteosarcoma sarcoma osteogenic cells (SaOs-2) [58]. They hypothesized however this loss in cell adhesion was due to an increase in temperature or a decrease in pH, not the SAW and US. When the temperature was maintained under 37°C, no significant cell detachment was indeed observed. In order to control the temperature, the duty cycle can be decreased, and the number of exposure cycles increased to deliver an equivalent dose to the cells in pulse rather than in a one-time exposure. However, a recent study showed that changing the number of exposure cycles had no effect on the observed cell detachment for similar exposure time and applied voltage [59]. Part of the remaining adherent cells in this study was destroyed through excess shear. Jötten et al. showed previously that the shear flow also impacted the cell detachment rate [52]. Other parameters are at stake such as the cell density, but also the cell type, rigidity, invasiness (etc.).

A study described different behaviors before detachment of red blood cells (RBC) depending on their pathological state [56]. For non-treated, non-infected RBC, the cell membrane translated, either rolling or flipping across the substrate before detachment. Glutaraldehyde-treated RBC showed a similar behavior but needed a longer period of time before detachment. Malaria-infected cells, on the other hand, adhered quite strongly for the duration of SAW exposure even if some cell translating, rolling and flipping were also observed. These findings pave the way for potential applications of SAW in diagnosis by use of biomarkers linked to mechanotransduction.

5.2. From cell manipulation to cell sorting

Single cell manipulation is a rising field at the intersection of biological sciences, microfluidics, and acoustics. SAW can be used to facilitate the cell collision with nanoparticles and induce cell lysis in very small volumes in microfluidic systems [60]. SAW can also guide cell seeding into porous scaffolds further than non-exposed cells [53,61]. A two-dimensional cell seeding pattern may also be built with possible spatial single-cell isolation [49]. Once isolated, single cells can be analyzed. Collins et al. showed that the US velocity gave information on the elasticity of the cells with 10^6 more sensitivity than atomic force microscopy (AFM). SAW could thus be used for the differentiation of cancer and healthy cells, as the elastic modulus might be a possible biomarker for invasiveness or metastatic potential.

High rate of cell manipulation and sorting can be achieved. A study in 2014 reached a sorting rate as high as 3000 cells.s^{-1} [54]. Under the influence of SAW, cells were sorted depending on their fluorescence. The used fluorophore (calcein-AM) was sensitive to cell metabolic activity and membrane integrity. The same year, a study realized a sorting of red blood cells by acoustic streaming depending on their infection states by the malarial parasite *Plasmodium falciparum* [48]. The authors noted that the cell density impacted their displacement within the shear flow. Noteworthy, powers above 250 mW did not lead to significant differences in cell behavior, while powers as low as 65 mW allowed efficient cell sorting. More recently, an attempt was made to sort first fluorescent polymer beads depending on their size, then brain cancer cells depending on their size and virulence [8]. Separation increased with the SAW cycle number. SAW induced more stability and flexibility in the cell sorting than standing SAW. Importantly, at the used power (126 mW) and frequency (39 MHz) the authors detected no significant effect on cell viability, proliferation and migration.

5.3. Wound healing: cell migration or proliferation?

In 2016, SAW were seen to enhance wound healing, with cells exposed to low powers (2 to 4 mW, at 159 MHz) for 48 h [57]. Osteoblasts (SaOs-2) were seeded as a monolayer with a zone of a few cm left empty: the "wound". After stimulation, the cells were faster to recreate a monolayer to join both side of the wound, hence the so-called "healing" process. Increasing the US intensity seemed to increase the healing process. No significant necrosis of the cells was observed. It remained unclear if the effect was due to an increase in cell migration or proliferation, and to mechanical or electrical stimulation. No preferred direction of migration or proliferation was detected; thus, the shear stress was supposed to be responsible for the wound healing intensification.

In 2020, Brugger et al. conducted a similar experiment, and confirmed the improvement in wound healing using SaOs-2 and canine and human kidney cells [7]. Here again, no morphological change and no oxidative stress were detected. This was observed however only if the flow stream was at a reasonable level, if too strong, cell detachment was observed, which coincides with earlier findings described in the section 4.1. on this review. The experiment answered that both cell migration and proliferation were enhanced, with a predominance of cell migration. Direct mechanical stimulation seemed to have more effect than electrical stimulation, but further studies are needed to confirm this hypothesis. The rise in temperature need to be controlled with an observed increased dependent on the used power: $\Delta T/\Delta P = 37$ K/W. Lately, Imashiro et al. reinforce Brugger's findings on cell migration, with a SAW system where the temperature was controlled to remain between 36 and 38°C, and the electrical stimulation was negligible as isolating layers of glycerol and PDMS separated the cells from the chip [62]. An increase by 28 and 42% of the cell migration speed was observed at 2 and 4 V, but the migration was suppressed at 18 V which corresponds to a 59,3 mW/cm^2 intensity. On the contrary to Stamp's study, they found a significant preferential alignment in the cell nuclei. They supposed it to be linked to changes in the cytoskeleton: increase in actin stress fibers and bundle thickness. The shear stress, estimated to 3.7 mPa, was supposed to be too low to induce such biological impacts rather due to the propagating acoustic waves themselves [51].

The question of whether cell proliferation could be enhanced by SAW is especially pertinent if we compared to the impacts of bulk US (paragraph 2.2.). SAW could increase human monocytes proliferation up to 36%, by using the following parameters: 49 MHz, 467 mW, duty cycle of 2.5% and 48 h exposure [50]. The temperature rise played no role, as they minimized the heating under 0.5°C by using a pulse stimulation with a 2.5% duty cycle. In this study however, the authors supposed that the acoustic streaming rather than the mechanical stimulation was responsible for the cell proliferation. Lower shear stress, without any US, have indeed been seen to increase the production of F-actin in human monocytes, inducing structural changes of the cytoskeleton that could lead to an increase

in proliferation. Considering their wide and crucial potential applications, new studies on cell mechanotransduction activated by SAW are expected in the next few years.

6. Towards experimental standardization

The study of the acoustic wave's effects on human and mammal cells is still a rising field. One major drawback is the lack of standardization between the published works. A main inconvenient is indeed the different expression of the US parameters: either the intensity, the power, or the voltage are given, especially in the most recent articles on HFUMS or SAW. Considering also the wide range of used cell types, comparing results from different studies is quite challenging. In Figure 6, an attempt was made to summarize the tendencies described throughout this review. Low frequency acoustic waves were seen to induced cell death due to cavitations, which, if used with optimized parameters, can perturb the cell membrane just enough to ease gene or protein translocation with the help of microbubbles. Low frequency US also mainly affect the cytoskeleton, with its fluidization and the formation of blebs. It either can be irreversible and induce cell death, or reversible and enhance cell proliferation and regeneration with applications in tissue regeneration. This is supposed to be due to acoustic pressure and resonance frequencies close to those of the cell components. At high frequency, the cavitation phenomenon is not observed, but the membrane permeability can still be boosted with applications in oncology, neurostimulation, or transfection of genetic material. Several studies showed stimuli of piezo ionic channels. If the mechanical transfers of energy start to be understood, much work is needed to assess the role of the temperature rise or the acoustic shear. SAW are mostly used for their potency in cell detachment, cell sorting and wound healing by increases of cell proliferation and/or migration. The cellular answers to SAW-induced US depends on the cell density and on properties such as the size or the elastic modulus. This allows the detection and separation of infection red blood cells for example. Most of these microfluidic manipulations are linked to the acoustic shear flow, as they were undergone under stabilized temperature. However, we have seen that even a slight change in temperature could impact the ion channel activation, thus further study on this parameter could be conducted. Moreover, the role of mechanic and electric stimuli in the biological response of the exposed cells are though still to be clarified.

Numerous parameters influence these results however: cell type, concentration, pathological state, US frequency, intensity, pulse mode, exposure time, and more globally the dose of exposure, as well as the environmental temperature and shear flow. Large scale studies, focusing on only one parameter and setting all others would be a solution to gain in understanding of the biological and physical mechanisms of action. This kind of work would feed on experimental standardization across the laboratories, efficient workload sharing, and systematic results dissemination.

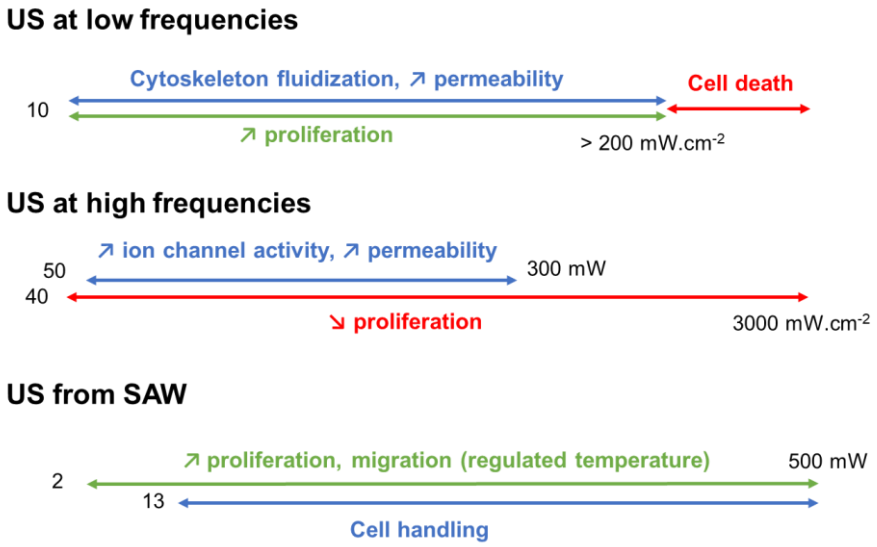

Figure 6. Sum up of the global tendencies of US effects on human and mammalian cells. Red: deleterious effects, blue: effects on the cytoskeleton and cell membrane, green: favorable effects.

7. Conclusion

This review summarized biological outcomes of human or mammal cells exposure to US, and tried to provide leads on the physical phenomena at stake. Even if not as emphasized with SAW, US can lead to cell death, damages, or decrease in proliferation if the parameters are not well adjusted. What is more of interest for cellular or medical sciences is the possibility to trigger an increase in cell proliferation, migration, and permeability either by inducing changes in the cytoskeleton or ion channel activity. Overall, standardized studies to assess the impact of each physical parameter shall be conducted to anticipate a specific cell line response to US and to design efficient microsystems for medical applications of acoustic waves.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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