Review Article

Opportunities from modulating ultrasound, from tissue ablation to tissue regeneration

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Abstract: The potential use of ultrasound for therapeutic purposes is known since the early decades of the last century. To date, only a few applications for clinical use are already a therapeutic reality, such as the ablative treatment of uterine fibroids, prostate cancer and palliative treatment for pain of bone metastases. Much more disparate are the ongoing biological research and clinical trial on the use of low and high intensity ultrasound predominantly, but not exclusively, in the oncology and neurologic fields. This review explores, in a systematic mode, the biological effects produced by modulating ultrasound beams from low to ablative intensities, revealing their enormous potential therapeutic implications. However, many efforts are still needed to translate these opportunities into clinical practices. Indeed, while ablation techniques need to be improved in sensibility and specificity of tumour and neurologic targets ablation, drug delivery and the other more fine cell killing methods need to be better studied through *in vitro* and preclinical researches, as a great variability of results are reported among similar type of experiments. Particularly, still there is the lack of the "acoustic dose" concept, adapted to each kind of biologic system, as the same ultrasound parameters can results in completely different responses, both for the lack of technical reproducibility in acoustic irradiation, both because the biologic response is tightly cell and tissue-type dependent. In this regard, the support of deep proteo-genomic analysis could help in the understanding of molecular signalling induced by ultrasound on specific biological models.

Keywords: Acoustic dose; biological effect; drug delivery; high intensity focused ultrasound (HIFU); low-intensity pulsed ultrasound (LIPUS)

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Introduction

The first studies on the biological effects of ultrasound (US) are dated at the beginning of 1900s, when the interaction between living tissues and high intensity and frequency sound waves were analysed (1). Then, in 1940 and 1950 William Fry and collaborators produced deep lesions in brains of cats and monkeys to study the use of focused US for therapeutic tissue ablation, but the lack of a precise imaging system limited their use till two decades ago (2-4). Nowadays, high intensity focused ultrasound (HIFU) systems, in combination with magnetic resonance imaging or diagnostic ultrasound, have given rise to novel therapeutic approaches like MR guided focused ultrasound surgery (MRgFUS) and US guided ultrasound surgery (USgFUS). These systems have become a new medical non-invasive therapeutic approach, thanks to their potentiality to obtain combinations of biological effects by modulating thermal, mechanical and chemical effects produced by the propagation of high and low energy waves through living tissues. The current HIFU clinical approved applications are finalized to the ablation of uterine fibroids, prostatic cancer and the palliative pain treatments of bone metastases. Moreover, different clinical experimentations are on-going worldwide in the field of solid tumours ablation, as well as treatments of neurodegenerative diseases, pain and vascular problems, to maximise the use of this technology (5). The last evolution of the addressing HIFU deep in the tissue

is the release of "sonication" from phased array transducers trough small ellipsoid-shaped spots. Multiple spots or "sonication" series, released under MR imaging, are needed to destroy a solid mass in the target volume (6). Although, its initial application has been the targeted non-invasive destruction of solid tumours, since US waves can penetrate the skin and tissue layer reaching a target inside the body, HIFU is also effective in tremor treatments and in arresting haemorrhage from either organs or vessels (7). Besides, novel therapeutic horizons become visible for the near future battle against cancer and neurologic diseases; since drug delivery, blood barrier opening, hyperthermia and cell sensitization to radiation treatments, modulating the waves beam intensity, render the use of US an adjuvant therapy in combination to traditional surgery, radiation or chemotherapy treatments (8). This review article would describe all the clinical and research potentialities deriving from modulating ultrasound beams in order to obtain the desired biological effect.

Physical and biological effects

Although the definition of "ultrasonic dose" is still debated, the main physical consequences caused by the ultrasonic waves' propagation across tissues are the thermal and the mechanical effects.

Thermal effects are the result of the tissue specific ability to absorb acoustic energy and can be easily monitored in clinical use by MR-thermometry (9). According to the final desired purpose, this effect can be utilized to induce hyperthermia, sensitizing tissues and enlarging membranes and junctions of normal tissue structures, or to provoke cells killing through the phenomenon called "coagulative necrosis", within ablative regimen. These two biologic effects can be reached alone or in combination, as the focused ultrasound can be directed deep in a target within the tissue, which is destroyed using a temperature above 56 ℃ for few seconds, while gradually lower temperatures are observed in the areas of tissues reached by lower energy intensities of the ultrasound beam (10). Specifically, when the temperature is lower than 100 ℃, tissues are subjected to the so called thermal fixation, in which cells do not undergo lysis and the tissue architecture remains relatively intact, but the cells are non-longer viable (11). Moreover, a sharp direct distinction is histologically visible between necrotic treated and not treated areas, while another less immediate biologic effect, following the ablative necrosis, is the release of a large repertoire of intracellular antigens that stimulate the immunological response (12). In addition to uterine fibroids and solid tumours, ablative temperatures reached by MRgFUS, can be used to develop new treatments for arteriovenous malformations and highly vascularized targets, obtaining thermal coagulation of blood vessels and safe and effective non-invasive bone metastases pain palliation, targeting of the periosteum area and thus resulting in bone denervation and pain relief. Furthermore, a pilot study translated this effect to facet pain alleviation, using low levels of energy to achieve a localized heating effect without damaging adjacent tissues, thanks to the high acoustic absorption and low thermal conductivity of the bone cortex (13).

On the other hand, working with sub-ablative temperatures give clinicians numerous possibilities of bio-effects and applications, the majority of which are under experimentation worldwide. For example, the use of hyperthermia in combination with radiotherapy or chemotherapy is a new object of research aiming to generate radio-sensitising or chemo-sensitising adjuvant treatments of target tissue (14).

Besides, among the mechanical effects produced by HIFU, a significant one is termed cavitation, which can be distinguished in non-inertial and inertial. The first one occurs when a gas-filled bubble, formed in tissue in response to US with high peak negative pressure, interacts with an ultrasound wave producing a stable oscillation of the gas filled bodies in response to positive and negative pressures of the US field.

The inertial cavitation occurs when oscillating bubbles undergo a violent collapse in response to US of specific frequency, pulse length, repetition frequency and pressure amplitude parameters. This collapse produces a rapid temperature and pressure increase that provoke cell lysis and the formation of reactive oxygen species (ROS). As consequence of this phenomenon, sub-ablative temperatures can be applied in presence of microbubbles, allowing the alteration of cell membrane permeability, which can facilitate drug or gene delivery, as well as the selective disruption of the blood-brain barrier (BBB) or blood clot. In particular, blood clot disruption is a new therapeutic opportunity, since maintenance of temperatures below the threshold required for ablation and acoustic cavitation, and can causes changes in endothelial membranes and the fibrinolysis cascade activation (15-17). From this perspective, the transcranial ultrasound application is a promising method of thrombolysis for the acute ischemic stroke treatment (18).

Another interesting mechanical phenomenon occurs when a wave is absorbed or reflected by a fluid. The radiation force due to the fluid moving under pressure produces an acoustic streaming, that causes a velocity gradient which in turn induces shear stress (19,20).

Table 1 Current commercial MRI and US guided systems

Type	Company	Device	Location
Magnetic Resonance	Insightec, Inc	Exablate 2000/2100; Exablate 4000	Tirat Carmel, Israel
	Philips Healthcare, Inc	Sonalleve	Boston, USA
	Profound Medical, Inc	Prostate System	Toronto, Canada
	Image guided Therapy, Inc	Targeted Fus	Pessac, France
Ultrasound	Chongqing Haifu (HIFU) Technology Company, Ltd	Haifu system	Chongqing, China
	US Hifu, LLC	Sonablate 500	Charlotte, USA
	EDAP TMS	Ablatherm	Vaulx-en-Velin, France

In addition, as the possibilities of modulating ultrasound offer a large and varied panel of combined bio-effect, histotripsy, worth to be mentioned. This new ultrasound ablation method depends on the initiation and maintenance till to cavitation of a bubble cloud to fractionate soft tissue, using short and high-intensity pulses of ultrasound (21,22).

Finally, opposed to the drastic killing effects observable under ablative regimen, regenerative properties have been *in vitro* and *in vivo* described in treatments using low-intensity pulsed ultrasound (LIPUS). These regeneration effects have been especially observed for bone and cartilage tissues, involving activation of osteoblasts, osteoclasts, chondrocytes, mesenchymal cells, with the exception of tissues already calcified (23).

Approved clinical employment

The great potentialities in the field of ultrasound' applications have driven, in recent years, a strong technological improvement in transducer design, energy delivery modes and real time imaging. Particularly, the modern MRgFUS systems represent a good technological combination of advanced acoustic transducers with the anatomic, functional, and thermal guidance of MRI, allowing accurate targeting, real-time temperature monitoring, and closed-loop control of energy deposition deep in the body (24,25) (*Table 1*).

Three types of devices have been developed: extracorporeal, intracavitary and interstitial. The first one is used to target tissues readily accessible from an acoustic window through the skin, such as uterine fibroids or breast. The second one is used for trans-rectal and trans-urethral treatments of prostate cancer or for intra-esophageal purpose; whereas the third one is dedicated to the treatment of the biliary duct and other difficult to access targets.

Moreover, the MRI temperature and imaging monitoring system offers far more accurate target details than US, in terms of tumour margin detection, surrounding anatomical details and temperature changes, which need to be monitored during therapy (26,27).

These features have brought the first FDA clearance, in 2004, for the MRgFUS clinical employment in the uterine fibroids treatment. Nowadays, the uterine fibroids and prostate cancer HIFU ablation plus the bone metastases pain palliative treatment is the only application with clinical acceptance. All other ablative treatments on breast, liver, kidney and other cancer targets represent on-going clinical trials.

The 10 years employment of MRgFUS for fibroids ablation in more than ten thousand patients, offers significant retrospective data to affirm that this minimally-invasive technique, results in very few patients reporting serious post-treatment complications and a comparable rate of success in terms of symptoms' reduction for medium sized fibroids and sensible improving of life quality and fertility preservation, compared to traditional surgical or radiological procedures (28-30). Moreover, although leiomyomas should preferably not exceed 10 cm in size, in 2012 Kim and co-workers introduced an interesting new technique for fibroid ablation, featuring one-layer ablation strategy for lesions larger than 10 cm (31).

Similarly, the prostate cancer ablation is, by now, a dated and consolidated technique that permits to reach the target with ultrasound beam, sparing organs at risk, such as the neurovascular bundle. In 2011, a long term study involving 803 patients, made evident the success of this technique in terms of 8 years overall (89%), cancer-specific (99%) survival rates and metastasis-free survival rate (97%) (32-34). Overall, this technique offers an excellent tumour control and complication rates (urinary or sexual dysfunction) comparable to traditional radiotherapy or radical prostatectomy interventions (35).

Finally, the bone metastasis pain palliation treatment, the third consolidated ablation technique, have sensibly and rapidly improved the life quality of bone metastatic patients, which can remain alive longer, providing them

fast and effective pain relief, without ionizing radiation, surgical intervention or serious side effects (36). Studies have reported rapid improvements in both visual acuity scale (VAS) scale and pain-measuring scales, just few days after the treatments, while in all studies no adverse events were recorded and all patients were able to reduce their medications at 3 months from MRgFUS (37,38).

A case report at Cefalù Hospital, Italy, described a 62-year-old patient with primary renal carcinoma, treated with MRgFUS (ExAblate System 2100, InSightec Ltd., Haifa, Israel) for the ablation of metastases sited on pelvic bone under the right iliac wing (39). The treatment was safe and effective in terms of pain reduction and a significant increase in life quality. Moreover, in this case, since the bone was completely eroded by the tumour, in addition to the mere palliative treatment, it was possible to exert high-energy sonications (5,500 joules) within the lesion. In correspondence of the treated area, weeks later it was observed the formation of new bone tissue, in the iliac wing and acetabular roof (39).

Experimental ablative protocols

Many clinical trials are on-going worldwide on the use of the MRgFUS technique for the treatments of solid tumours, for instance breast, liver, pancreas and other abdominal targets, as well as brain cancer and neurological disease treatments.

Nonetheless the efforts made by researchers, the effectiveness and safety of this technique have not yet reached an adequate level of quality to overcome the standard therapeutic protocols (40). The main problem to solve is to achieve a 100% of tumour ablation without positive margins, since a small percentage of cases still show peripheral residual tumour presence, most probably due to a sensitivity limit of the MRI imaging system. Therefore, current experimental MRgFUS ablative protocols include the surgical removal of tissue after ablation.

The group of Gianfelice was the first to report the results of a "treat-and-resect" protocol type applied to two groups of 12 and 17 invasive breast cancer patients, confirming a mean of 88,3% of ablated tumours and the need for larger (>5 mm) safety margins around the MRI visible tumour (41,42). The group of Furusawa recently reported better results on 30 breast cancer patients, using the same type of "treat-and-resect" protocol. In this case, the mean percentage of tumour necrosis was 97% and 50% of patients had 100% necrosis of the ablated tumours (43). Again, Gianfelice and co-workers have described, in 2003, a method to assess the ability of dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) parameters [increase in signal intensity (ISI); maximum difference function (MDF); and positive enhancement integral (PEI)] to monitor residual tumours following MRgFUS treatment of breast cancer. This method achieved a sensitivity of 77% and a specificity of 100% (41,42).

Besides the difficulty of getting 100% of ablation with negative margins, other critical issues on the use of this methodology for the treatment of abdominal targets, such as liver, kidney and pancreas are under investigation (44). Particularly, in these cases the problems are related to the acoustic windows, restricted by the presence of rib cage and bowel, which can distort the ultrasound beam. Intestinal gas presence can cause reflection and unwanted heating, preventing ultrasound energy from reaching the target. Furthermore, the organ motion is another problem to solve, on which different groups of engineers are working worldwide. One solution could consist in focusing other target points, such as blood vessels, close to the organ to treat, as proposed by Ross and colleagues in 2008. In this case the sub-pixel tracking accuracy was measured to be 5.7 ms (SD: ±1.6 ms), sufficient for a real-time use (45).

However, experimental protocols for abdominal cancer ablation have been on-going for the last ten years, with the purpose of tumour control and pain palliation. A recent work of the group of Anzidei conducted on seven selected patients with unresectable primary pancreatic adenocarcinoma showed a successful procedure in 6 out of 7 patients, since in a single patient, the lesion accessibility was limited at treatment time and the procedure was suspended. Follow-up imaging revealed negligible (n=1) or no (n=5) tumour regrowth within the ablation area (46). In 2005, a study reported the results of treatments on 30 patients with hepatic or renal tumours, according to four trial protocols. HIFU exposure resulted in discrete zones of ablation in 25 out of 27 evaluable patients (93%). Ablation of liver tumours was achieved more consistently than that of kidney tumours (100% *vs.* 67%, radiologically assessed). The adverse event profile was favourable when compared to more invasive techniques (47).

Clinical trials on other oncologic disease are in progress, like treatment for osteoid osteoma, suggesting that MRgFUS treatment can be performed safely with a high rate of success and without apparent treatment-related morbidity (48).

Last but not least, MRgFUS is employed in the neurological field, with application to brain tumour surgery and brain disorders treatments of neuropathic pain, essential tremor and Parkinson disease (49,50). This technology has introduced revolutionary treatment for neuro interventions,

due to its ability of precision lesioning, supported by imaging technologies able to visualize the anatomy within the skull, without the need of a craniotomy and a real-time temperature control that allows to safety achieve targets close to nerves, cortical and subcortical regions, and other critical structures.

For decades, therapeutic ultrasound through an intact skull was considered impossible due to disruption of the focused acoustic beam and skull heating. In the 1950's William Fry applied HIFU to the human brain with the intent to create discrete lesions to treat hyperkinetic disorders such as Parkinson's disease. Today, both Insightec Ltd and SuperSonic Imagine (Aix en Provence, France) developed an MRgFUS system for brain treatment. The Insightec system, termed Exablate 4000, is in a phase I clinical trial for the treatment of primary and metastatic brain malignancies. The hemispheric phased arrays around the head allow the focus restoring by adjusting each phased-array element according to the thickness of the underlying bone (51,52). Clinical studies are on-going on over 130 patients with neuropathic pain, essential tremor, Parkinson's disease and obsessive-compulsive disorder, showing very promising results and immediate improvements after treatments (53-55).

In addition, pre-clinical studies, investigating the possibility to combine neuro-functional ablative and non-ablative methods, represent a precious tool to achieve the near-future expansion of MRgFUS treatment in new fields, such as BBB disruption and drug delivery.

Biological effects of ultrasound, a weapon for future ultrasound therapeutic uses

At present, a conspicuous number of potentially interesting US applications, which do not rely solely to the direct tissue destruction, are currently on-going *in vitro* and preclinical research subjects. These topics are interesting, not only from a purely scientific point of view, but reserve promising potential applications for the introduction of revolutionaries and personalized therapeutic interventions in the next decades.

However, despite the biological research is very intense on several fields, in this proteogenomic era, there is still the need for a deep comprehension of molecular mechanisms sustaining the biological effects of ultrasound. To this aim, the *in vitro* research is a fundamental step to reach the more advanced pre-clinical studies.

In order to comprehensively discuss the wide types of obtainable biologic effects obtainable in the near future, we will classify them according to the ultrasound power ranges, from the low intensity ultrasound to the ablative powers (*Figure 1*).

The treatments with LIPUS use energy intensities of the order of mW to 3 W/cm^2, pulsed at repetition frequencies ranging from 0.5 to 100 Hz, with a pulse width of milliseconds and frequencies around 1 MHz (56). Their benefits are widely recognized in the field of bone and cartilage regeneration, stimulating fracture healing. The mechanisms by which ultrasound can trigger these effects remain poorly understood, nonetheless the events of bone regeneration are well known and physiologically distinguished in an early inflammatory phase, a reparative phase and a late remodelling phase, sustained by the involvement of multiple factors, including cytokines, hormones and growth factors, released in the extracellular matrix (ECM) and interacting with different cell types (mesenchymal stem cells, endothelial cells, bone and cartilage cells) recruited to the site of tissue damage.

Several pathways have been recognized to be activated by LIPUS, such as TGFβ signalling and MAPK signalling. However, the diversity of current experimental set-ups renders heterogeneous the results of *in vitro* studies, therefore the overall evaluation of biological effects induced by LIPUS needs the design of dedicated experimental set-ups, in which the different mechanical phenomena can be controlled (57). Furthermore, among the observed effects on tumours, low intensity ultrasound act as modulators of host-tumour response, whereas a study showed that LIPUS can also induce DNA damages, as demonstrated by the presence of gammaH2AX-positive foci in leukaemia cells (58,59). In addition, important evidences suggest that the application of low and sub-lethal intensity ultrasound is able to disturb the balance between survival and apoptosis/cell death. The ability to induce cell death in the absence of necrosis represents a novel approach of apoptotic cancer therapy. Several *in vitro* studies are evaluating this issue to induce stress response and apoptosis with minimal lysis in several cancer cell lines or to use this approach in conjunction with hyperthermia, photodynamic therapy, radiotherapy and chemotherapy to produce a synergistic effect (15,60,61). In a work conducted by the group of Feril in 2002, the effects of ultrasound on hyperthermia-induced apoptosis was studied on human lymphoma U937 cells, exposed to 44.0 degrees for 10 min. and continuous 1 MHz ultrasound at intensities of 0.5 or 1.0 W/cm^2, considered non-thermal energies and sub-threshold for inertial cavitation. They observed that 0.5 W/cm^2 in combination with hyperthermia synergistically induced apoptosis, whereas at 1.0 W/cm^2 with hyperthermia an augmented instant cell lysis without significant change in apoptosis

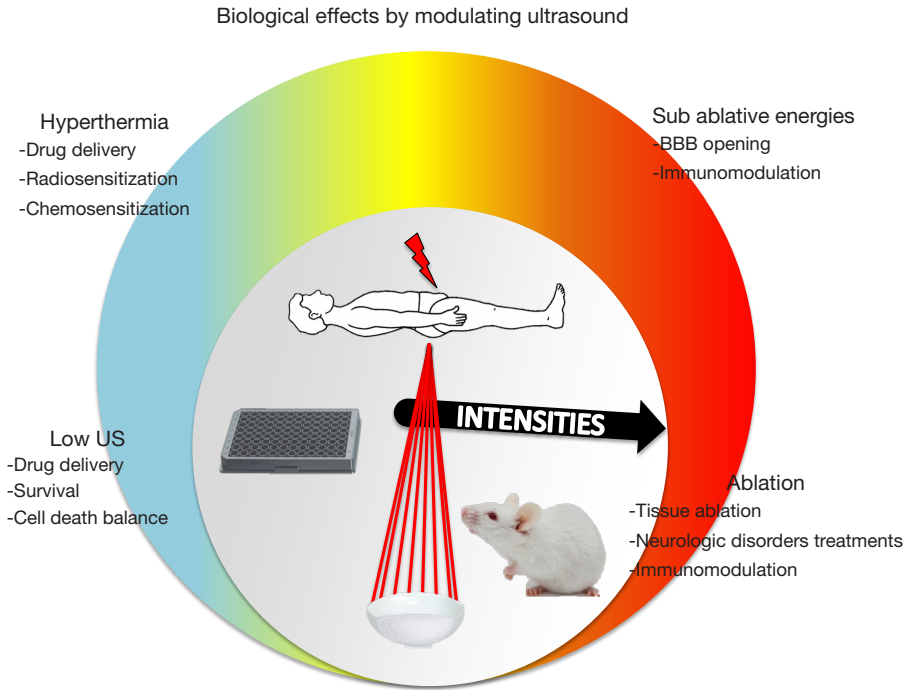

Figure 1 A schematic description of biological effects observable in dependence of US intensities and duty cycles increasing. A route across all the possible ultrasound application on *in vitro* and *in vivo* models in research and clinical field. US, ultrasound; BBB, blood-brain barrier.

ratio was showed (62). Furthermore, in a work conducted by the same group in 2008, the networks activated by U937 cells subjected to LIPUS treatment (0.3 W/cm^2 for 1 min) has been analysed by global-scale microarrays. Six hours later, apoptosis without cell lysis was observed. The networks of down-regulated genes regarded cell growth and proliferation, gene expression or cell development, while the ones of up-regulated genes resulted associated to cell movement, cell morphology and cell death (63). In addition to that produced by the simple ultrasound treatments, the cell death can be amplified by the contemporary administration of cytotoxic molecules, drugs or genetic material, entering in transiently permeabilized cells by different drug delivery methods, world-wide under study (64).

Although it has to be taken in mind that each biological response to a kind of physic ultrasound setting is, always, cell-type dependent, the mechanisms underlying the transient membrane permeability can be summarised like the following: (I) low intensity ultrasound leading to stable cavitation of microbubbles; (II) high intensity ultrasound leading to inertial cavitation with microbubble collapse; and (III) ultrasound application in the absence of microbubbles. More precisely, using low intensity ultrasound, the endocytic uptake of several drugs is stimulated, while short but intense ultrasound pulses can be applied to induce pore formation and direct cytoplasmic drugs uptake. Hyperthermia effect, with temperature increase between 39 and 41 ℃, can also be commonly used alone or synergistically to mechanical effects, for the purposes of drug delivery and can be combined with the use of temperature sensitive liposomes (LTSLs) (65).

Ultrasound intensities can be adapted to create pore sizes correlating with drug size. Larger drugs, such as nanoparticles and gene complexes, will require higher ultrasound intensities in order to allow direct cytoplasmic entry or the use of engineered delivery systems, while small molecules are able to diffuse passively through small pores created at lower ultrasound intensity.

Additionally, the expressions of transgenes can be placed under the control of temperature sensitive promoters, such as those of heat-shock genes.

In a work of Zhong and colleagues the biological membrane opening is shown using a direct approach, by means of electron microscopy. In this way it was possible to observe the formation of pores of diameter between 0.1-0.5 μm following a sonoporation experiment in presence of microbubbles. In particular, the pores are detectable 3 seconds after exposure

to ultrasound (parameters used: 1 MHz frequency, 10% duty cycle, 1 kHz repetition rate pulsed, 0.5 MPa peak of negative pressure, in presence of 1% microbubbles) (66). After one minute, the microscopic observation shows that pores were repaired and the membrane was covered with "patches" as protuberances dispersed along the membrane surface. This mechanism appears to be transient, since 1 h after treatment the membrane surface is already more smooth, a sign that cells attempt to return to their original shape.

Instead, the using of grater frequencies can cause cavitation and produce tissue damage if not properly controlled. To overcome this problem, many studies use protocols with pulsed sonication frequency of 1-3 MHz and intensity of 0.5-2.5 W/cm^2 (67). However, these conditions are not always very efficient and the improvement of drug delivery protocols needs to be adapted case by case, considering the cells and tissue type under treatment and the type of molecules to delivery. Today, thanks to advances in ultrasound control techniques, many studies are carried out to evaluate the combined effects of ultrasound and chemotherapy and a lot of effort is currently made in the optimization of drug delivery particles that can be addressed in a targeted or non-targeted way. Polymeric nanoparticles, microbubbles and several types of liposomes can be engineered to contain genetic material, drugs or other cytotoxic molecules, that can be locally released under ultrasound control, taking advantage from inducing cavitation or local temperature increase when the LTLs or similar systems are used. Also, they can be specifically delivered combining them with antibodies recognizing specific cell antigens, so that only the targeted cells will be treatment, sparing the surrounding tissue and decreasing the risk of side effects (68-73).

These experimental approaches represent a challenge for numerous cancers and diseases, which today have low chance of success. For example, the efficacies of chemotherapeutic agents are severely restricted in the brain, due to the BBB, that prevents large molecules from penetrating into the parenchyma from the brain vasculature. If focused ultrasound is applied in the presence of microbubbles, the BBB can be temporarily disrupted, allowing the penetration of large molecules to reach image-selected regions of the brain. Kinoshita M. and collaborators demonstrate the ability to drive large-molecule drugs like Herceptin (trastuzumab) and doxorubicin through the BBB without damages to brain tissue, which may allow the use of these drugs to treat primary and metastatic brain tumours (74). The group of Yang reported a BBB permeability increase in rats inoculated with F98 glioma cells within the brain, treated with pulsed-HIFU. The effect of sonication resulted in an accumulation of Evans Blue dye (used as a marker that bounds to albumin, a complex which reaches a molecular weight of about 68.0000 Da), approximately 2-fold higher in the region of the tumour treated with HIFU compared to the contralateral part of brain (75).

The group of Jordão in 2010 has carried out experiments of HIFU mediated drug delivery on murine models of Alzheimer's disease, using an anti-amyloid β antibody, injected in combination with a MRI contrast agent. The authors describe the antibody binding to the plates within minutes from the treatment and reported that the bonds remained visible for at least 4 days, reducing the pathology in terms of number, size and mean of plaque surface area. These early results suggest that focused ultrasound is able of BBB-opening in a reversible, localizable, and non-invasive manner (76).

As regard to other kinds of therapeutic combination with ultrasound, in 1979, already Sapareto and colleagues have explored the field of hyperthermia and radiation combination therapy, in order to sensitize cells to radiation effectiveness. These *in vitro* studies demonstrated both an optimum temperature range and a practical split thermal treatment method, which provide maximum interaction of heat and radiation in terms of cell death (77).

The effect of radio-sensitization induced by hyperthermia can be attributed to the fact that heat is a pleiotropic damaging agent, altering protein structures and the DNA damage repair (78). Indeed, heat does not induce DNA double-strand breaks but rather appears to inhibit or just delay the DNA damage repair. For example, hyperthermia enhances the IR-induced ATM kinase activity producing the ATM-dependent phosphorylation of H2AX, altering the chromatin structure. Moreover, heat causes protein unfolding, which can lead to protein precipitation and sequestering of the proteins involved in DNA damage repair. Thus, hyperthermia influences several molecular parameters involved in sensitizing tumour cells to radiation and can enhance the potential of targeted radiotherapy (79).

At the end of this long enumeration of ultrasound applications of such disparate biologic effects, it worths mentioning the ability of the ablative process to activate and enhance an anti-tumour immunologic response.

In fact, death by necrosis, rather than apoptosis, damage cells in a more destructive way, allowing the release of intracellular molecules, orienting the immunological response and miming the effect of a natural anti-cancer vaccine, through the modulation of lymphocyte subpopulations (Th1, Th2, Natural Killer, B), sets of cytokines, chemokines

and pro-anti-inflammatory molecules towards a target to eliminate. This effect has interesting implications, since cancer cells develop the ability of immuno-surveillance "escaping" during neoplastic progression (80). In this way, after the ablative treatment and the release of tumour-specific intracellular signals, the immunological response against tumour targets is enhanced reducing the risk of metastasis and recurrence. The group of Wu observed an increase in the CD4+ cell population and the CD4+/CD8+ ratio after HIFU treatment in a group of patients with solid tumours (six patients with osteosarcoma, five with hepatocellular carcinoma and five with renal cell carcinoma) (81). Moreover, in patients showing altered lymphocyte percentages before treatment, a re-establishment of normal conditions was observable already a few days after treatment. The group of Xu conducted a similar study on 48 patients with breast cancer undergoing mastectomy, randomly divided into a control group (25 patients) and in a group subjected to thermal ablation with HIFU (23 patients) 1-2 weeks prior to surgical resection. The study has shown that in the resected tumours treated with HIFU a significant increase of DCs, macrophages and B cells infiltrates was observable along the margins of treated regions (82).

Conclusions

This review has enumerated a long list of biologic effects induced by ultrasound, each one having enormous potential future therapeutic implications. However, many efforts are still needed to translate these opportunities into clinical practices. Indeed, while ablation techniques need to be improved in sensibility and specificity of tumour and neurologic targets ablation, drug delivery and other more elegant cell-killing methods need to be better investigated through *in vitro* and preclinical studies, as variable results are reported among similar type of experiments. In this regard, biologists and physics need to better refine the concept of "acoustic dose" adapted to each kind of biologic system, as the same ultrasound parameters can result in completely different responses, both for the lack of technical reproducibility in acoustic irradiation and the cell and tissue-type dependence of biologic response. Particularly, the aim of shifting the delicate survival/cell death balance, for the purposes of cell killing, needs the support of deep highly throughput technologies, which could facilitate the comprehension of molecular signalling induced by ultrasound and the identification of biological markers of interest in targeted therapies (83). Our group of physics and biologists is working in this direction, for the realization of reproducible performances of *in vitro* acoustic irradiation and the molecular characterization of tumour responses to combined ultrasound and cell killing sensitizing type of treatments.

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