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ROLE OF BIO-RESONANCE FOCUSED ULTRASOUND ON STEM CELL PROLIFERATION AND GROWTH: A REVIEW

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ABSTRACT

Changes in critical cellular functions caused by therapeutic ultrasound (U.S.) are likely to be involved in the effects of therapeutic ultrasound (U.S.) on tissue healing processes in vivo. The effects of a single 5-minute C.W. 3.00-MHz U.S. exposure on the growth and functional activity of a human osteoblast-like cell line (MG63 cells) and human periodontal ligament (PDL) cells in vitro were investigated in this study. Although average spatial intensity (ISA) values ranging from 140 to 990 mW/cm2 were found not to affect cell proliferation, flow cytometry (FCM) analysis revealed that there were significant and distinct effects on cell function. As a result, bone-associated proteins in MG63 cells were downregulated, while collagen type I (COL I) was unaffected, and fibronectin (F.N.) was upregulated at low levels. In PDL cells, however, bone protein expression was dose-dependent, and the levels of F.N. and COL I were reduced. These findings suggest that the United States significantly impacts connective tissue cells' functional activities in vitro, impacting tissue repair and regeneration in vivo.

KEYWORDS: Ultrasound, Bio-resonance, Bone cells, Periodontal ligament cells, cell growth, cell repair, cell proliferation.

INTRODUCTION

Ultrasound (U.S.) was widely used for diagnostic purposes, and many soft tissue radiological examinations were performed using it. Despite the overwhelming clinical value of the U.S. as a diagnostic tool, there is growing interest in US-induced biological effects that have shown the potential to damage and stimulate tissues. In vivo, harmful biological responses such as thermal lesions (Clarke and ter Haar 1997), lung hemorrhage, and hind limb paralysis have all been demonstrated (Frizzle et al. 1994). Cell membrane damage, cell survival, and cell lysis were also reported in cell-level studies (Fahnestock et al. 1989). (Dooley et al. 1983). Sister chromatid exchange was also extensively researched for the possibility of genetic damage, though the results were inconclusive (Stella et al., 1984; Ciaravino et al., 1986).

On the other hand, many studies have suggested that therapeutic ultrasound can have beneficial effects in certain circumstances, leading to the development and widespread use of therapeutic ultrasound by physiotherapists for various soft tissue injuries, including tendon and ligament injuries (ter Haar et al. 1987). Soft tissue studies using in vivo physiotherapy have shown improved healing rates (Byl et al. 1992) and tissue strength (Enwemeka et al. 1990). In vitro, such

therapeutic levels had stimulatory effects, including increased protein synthesis (Edmonds and Ross, 1988) and calcium intake (Edmonds and Ross, 1988). (Mortimer and Dyson 1988).

Furthermore, several common wound sites, such as venous ulcers (Johannsen et al. 1998), lateral epicondylitis (tennis elbow) (Binder et al. 1985), and bone fractures, in particular, have shown a beneficial U.S. response (Kristiansen et al. 1997). Thus, double-blind, placebo-controlled studies of human fractures treated in the United States revealed improved healing rates of 30–40%. (Kris Hansen et al. 1997; Heckman et al. 1994). Other in vivo studies found significant improvements in fracture healing at continuous-wave average spatial intensity (ISA) values of 30–100 mW/cm2 (Pilla et al. 1990; Yang et al. 1996; Zorlu et al. 1998). Although Tsai et al. (1992) found a deleterious response at 1000 mW/cm2, stimulation of bone healing was also demonstrated at relatively higher ISA levels, such as 500 mW/cm2 (Dyson and Brooks 1983). A recent in vitro study in mouse osteoblasts (Kokubu et al. 1999) found increased production of prostaglandin E2 with relatively low-intensity exposure (ISA of 30 mW/cm2), suggesting this as a possible mechanism in U.S. fracture healing. However, few other studies have looked into the precise effects of the U.S. on

fundamental cellular and molecular processes involved in the repair and regeneration of either hard or soft connective tissues, limiting the development of new strategies that could improve damaged tissue wound healing. Because of the widespread use of connective tissue damage clinical therapy in the United States, the country is likely to impact connective tissue cells' critical functional activities significantly. As a result, the current study used flow cytometry (FCM) to accurately measure the effects of the United States on the production of specific extracellular matrix (ECM) proteins at a frequency and intensity range that was representative of current clinical therapeutic doses (ter Haar et al. 1987). The components chosen were osteonectin (ON), osteopontin (O.P.), and bone sialoprotein (BSP), all of which are closely linked to bone function and integrity, as well as fibronectin (F.N.) and collagen type I (COL I), which are both widely distributed in all connective tissues.

Role of Bio-Resonance Focused ultrasound on Cell Growth and Repair: A Review
Effects of ultrasound on cell proliferation
Cell growth in response to the U.S. was measured using direct cell counting, as described in the Materials and Methods section. Compared to the growth of non-exposed cells used as controls, the proliferation rates of MG63 and PDL cells after exposure to increasing doses of U.S. are shown in Fig. 2. MG63 cell growth was reduced on day two and increased on day three after insonation (Fig. 2a), but these differences were not statistically significant ($p . 0.05$). Increased U.S. doses did not appear to significantly affect MG63 proliferation (at days 3 and 4). At all U.S. intensities, the PDL cells' growth (Fig. 2b) was statistically identical to that of the nontreated control cells ($p . 0.05$).
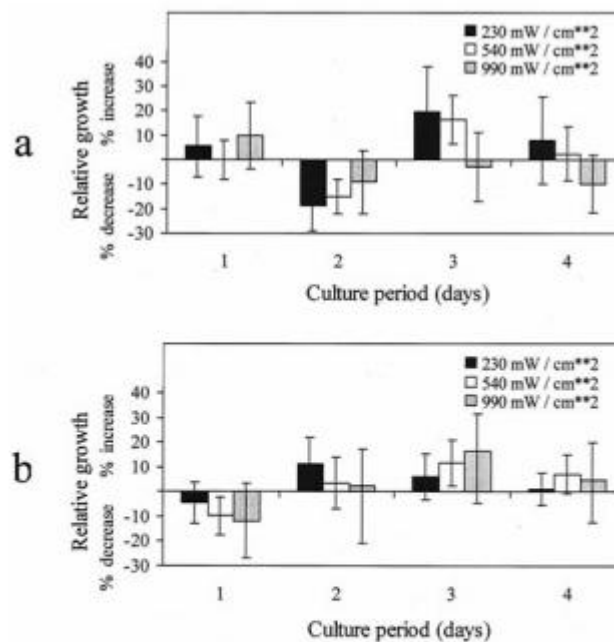

Fig. 2. Growth of (a) MG63 cells and (b) PDL cells following exposure to US. The rates of proliferation are shown relative to the control cells on each day following exposure to 230, 540 and 990 mW/cm^2 intensities of US. There was no significant difference in growth at any intensity level, compared with the respective nonexposed control cells ($p > 0.05$). Vertical lines are $\pm$ SE.

Effects of ultrasound on antigen expression
Figure 3 shows F.N. expression in MG63 and PDL cells in a representative FCM experiment. These findings show that the lowest ultrasound dose (140 mW/cm2) upregulates F.N. significantly (compared to control cells) and to a lesser extent at 540 mW/cm2, but not at the other two doses (Fig. 3a). However, in this experiment, all U.S. intensities appeared to reduce PDL cell expression (Fig. 3b) significantly. The response of the bone-associated proteins ON, O.P., and BSP and the soft

connective tissue associated with antigens F.N. and COL I was very noticeable in MG63 cells, as shown in the results of all FCM experiments in Fig. 4a. Thus, ON expression was down-regulated in the former at all intensities, reaching significantly lower levels at the highest and lowest doses (p, 0.05), and O.P. expression was dose-dependent, reaching a significantly lower value of 40% of control cells at a maximum dose of 990 mW/cm2 (p, 0.05). BSP was significantly reduced to approximately 50% of control values (Fig. 4a).
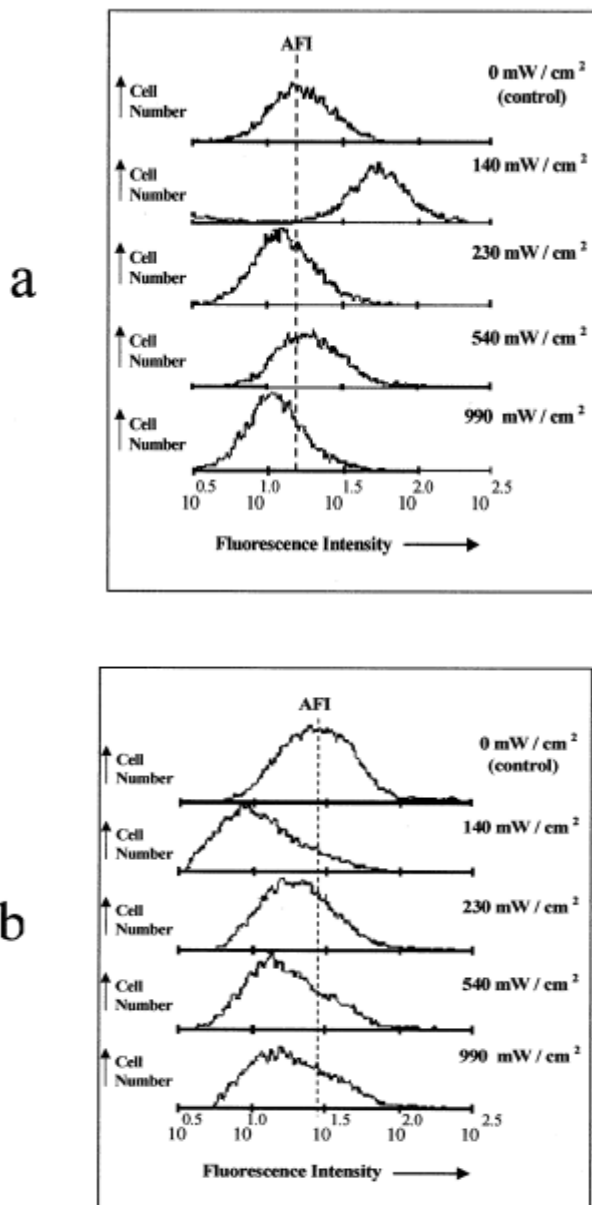
Fig. 3. Representative FCM analysis of FN expression by the (a) MG63 cells and (b) PDL cells 5 days after US exposure at 140, 230, 540 and 990 mW/cm². In (a), note the up-regulation of FN at a low intensity (140 mW/cm²) and, in (b), the reduced expression of this antigen by the PDL cells at all US intensities, compared with the nonexposed control cells.

F.N. expression by MG63 cells, on the other hand, was upregulated at both the lowest and highest intensities (200 percent and 150 percent control at 140 and 230 mW/cm2, respectively). However, the differences were not statistically significant (p . 0.05). COL I, for example, was unresponsive to all U.S. intensities, as shown in Fig. 4a. Although there were notable differences between the effects of the U.S. on each of the bone-associated proteins and between F.N. and COL I, the response of PDL cells to the U.S. was different from that of MG63 cells (Fig. 4b). While ON expression was gradually down-regulated as U.S. doses were increased (22 percent lower than control at 990 mW/cm2), O.P.

expression was firmly and significantly elevated at the highest dose, reaching a level 38 percent higher than control cells. However, there was no clear pattern of BSP expression modulation in response to increased U.S. doses, as shown in Fig. 4b, as this antigen was reduced at the two intermediate levels but remained unchanged at the highest and lowest U.S. doses. On the other hand, it was found at all four intensities, with a maximum and statistically significant drop of 35% at 230 mW/cm2. Similarly, at 140, 230, and 540 mW/cm2, COL I was significantly reduced in PDL cells by about 20% of the control level (p, 0.05), though it was only reduced by 6% 990 mW/cm2.
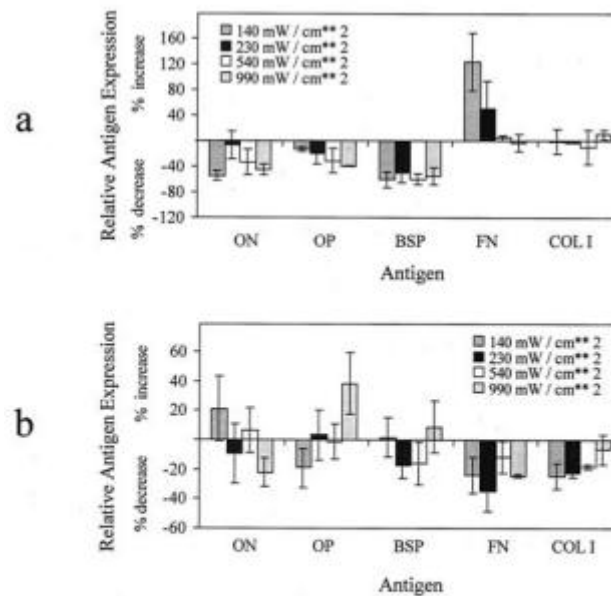
Fig. 4. Effects of ultrasound on expression of connective tissue antigens by the (a) MG63 cells and (b) PDL cells, 5 days after exposure. The data show the relative expression of each antigen in response to a range of US intensities, as described in Fig. 3, compared with nonexposed controls. Note the antigen- as well as cell-specific responses to US. Vertical lines are ± SE.

DISCUSSION

For soft connective tissue injuries, ultrasound treatment of wound sites is now standard (ter Haar et al. 1987), and U.S. repair of hard connective tissue damage has also shown promise in clinical trials (Kristiansen et al. 1997) and in vivo studies (Pilla et al. 1990). Although the U.S.'s precise cellular effects are unknown, the U.S. likely has a significant impact on the fundamental processes that promote repair and regeneration of soft and hard connective tissue. Studying biological responses in the United States is critical not only for developing safe diagnostic ultrasound guidelines (WFUMB 1998) and improving, developing, and implementing effective therapeutic strategies (Dyson 1987). The current study looked at the effects of different U.S. intensities on specific connective tissue components in vitro. These have previously been shown to play an important role in the structure, integrity, and function of the ECM and wound healing and tissue remodeling. Osteoblasts and fibroblasts were chosen as "target" cells because they make up a large portion of hard and soft connective tissue, and they responded well to the therapeutic U.S. in histological studies (Singh et al. 1997). The acoustic parameters used, which range from 250 to 1000 mW/cm2, are similar to those previously used in clinical physiotherapy (ter Haar et al. 1987), and the lowest intensity value, 140 mW/cm2, is close to "worst-case" B-mode diagnostic exposure (Henderson et al. 1995). Even though the intensities used in this study had little, if any, beneficial effect on human cell proliferation in culture, all U.S. doses selectively influenced ECM antigens' expression by MG63 and PDL cells. Consequently, while MG63 cells reduced the expression of the bone-associated proteins ON, O.P., and BSP at all doses, the response of these antigens in PDL cells was variable, and in some cases, such as ON and O.P., was upregulated at some doses. F.N. expression was also significantly upregulated in MG63 cells but blocked in PDL cells, while COL I expression was unaffected in MG63 cells but down-regulated in PDL cells. Our findings that F.N. expression in MG63 cells and ON expression in PDL cells were significantly upregulated at the lowest intensity (140 mW/cm2) are consistent with low-level stimulation reports in vivo at 100 mW/cm2 (Yang et al. 1996; Zorlu et al. 1998) and low-level reports (30 mW/cm2) with increased exposure times and multiple applications (Yang et al. 1998). (Pilla et al. 1990; Heckman et al. 1994; Kristiansen et al. 1997).

Dyson and Brookes (1983) reported such stimulation at 5-minute exposures at intensities up to 500 mW/cm2, though attenuation-correction for overlying tissue will have reduced the actual intensity applied. The mechanisms underlying these differential changes in ECM component expression are still unknown, but the U.S. has previously demonstrated a strong influence on the plasma membrane's integrity and transport properties (Dinno et al. 1989). As a result, the effects we saw may be due, at least in part, to modulation of post-transcriptional processes that affect the cell's secretion of connective tissue antigens. As previously reported, studies using the drug monensin, an intracellular transport inhibitor that blocks secretion, will help assess such changes (Bou-Gharios et al. 1994; Kuru et al. 1998). However, the effects we observed were relatively long-lasting, implying that cell function was fundamentally altered; research is currently underway to

determine whether the corresponding ECM genes' transcriptional activity is a direct target of the U.S. The exposure method used in this study had the advantage of exposing adherent cell types in standard cell culture flasks. From quoted dosimetry measurements, exposure by an intervening polystyrene layer resulted in a 14 percent intensity loss. Plastic attenuation would account for a large portion of this total, with the remainder lost through reflection at each fluid-polystyrene interface. As a result, less than half of the 14 percent lost intensity could be reflected at each interface, making significant standing wave activity in the flask unlikely.

Furthermore, the polystyrene layer only caused a minor heating effect on the monolayer surface, with a maximum dose value of 0.5°C and lower doses of 0.2°C or less (data not shown). These temperature variations are similar to those that occur naturally in tissue culture incubators in vivo and in vitro cells and are unlikely to cause the U.S. effects observed in this study. As a result, these findings raise the possibility that nonthermal mechanisms are involved in cell function changes induced by the United States. Using in vitro isolated cells and cell lines, the FCM technique was able to investigate precise cellular responses to the U.S. for the first time and deliver different and well-characterized ultrasonic doses, which is challenging to achieve in vivo. When comparing soft and hard connective tissues with different acoustic properties and transmitting different intensity levels to in vivo component cells, this is especially important. Furthermore, cavitational and shear processes in tissue culture cells are generally superior to those in body tissues (WFUMB 1998). Further research is needed to determine the cavitation and radiation forces within culture flasks to assess their effects on biological responses.

CONCLUSION

In conclusion, this study demonstrates that the United States impacts critical in vitro production of ECM antigens and, as a result, the functional activities of these cells. Although it is difficult to extrapolate the current in vitro findings to the clinical situation, the differential cellular responses to the U.S. suggest that using specific and different U.S. parameters could help achieve optimal therapeutic efficacy in vivo at hard and soft connective tissue sites. Moreover, despite the need to be cautious about extrapolating in vitro results, our findings suggest that the FCM technique could help determine the potential benefits and drawbacks of U.S. exposure at the clinical level. This could help boost the efficacy of therapeutic ultrasound in wound healing and tissue repair.

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