Pulsed-low intensity ultrasound enhances extracellular matrix production by fibroblasts encapsulated in alginate

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Abstract
In this study, the effect of pulsed-low intensity ultrasound on cell proliferation, collagen production and glycosaminoglycan deposition by 3T3 fibroblasts encapsulated in alginate was evaluated. Hoechst 33258 assay for cell number, hydroxyproline assay for collagen content and dimethylamine blue assay for glycosaminoglycan content were performed on samples from cell cultures treated with pulsed-low intensity ultrasound and a control group. Pulsed-low intensity ultrasound shows no effect on cell proliferation, while collagen and glycosaminoglycan contents were consistently higher in the samples treated with pulsed-low intensity ultrasound, showing a statistically significant difference ($p < 0.05$) on day 10. Alcian blue staining showed that glycosaminoglycans were deposited around the cells in both groups. These results suggest that pulsed-low intensity ultrasound shows no effect on cell proliferation but has potential for inducing collagen and glycosaminoglycan production in cells cultured in alginate gels.

Keywords
Alginate, fibroblast cells, collagen, ultrasound, glycosaminoglycan

Introduction

Ultrasound (US) is widely used in clinical practice as a diagnostic, surgical and therapeutic tool.[1] In the case of therapeutic US, its biological effect can be classified as thermal or non-thermal. The frequency and intensity can be varied depending on the application.[2,3] The thermal effects are caused by high-intensity therapeutic US, which generates heat.[3,4] In contrast, low-intensity US has non-thermal effects caused by cavitation and acoustic microstreaming, leading to most of the energy transferred to biological tissues being mechanical.[3,5–7] When compared to biochemical stimulation, physical stimulation using US offers several advantages. Physical stimulation is non-toxic and has a wide margin of biological safety.[8] Furthermore, it can be used alone or in conjunction with biochemical stimulation.[8]

Pulsed-low intensity ultrasound (PLIUS) has a low intensity (less than 1 W/cm²) and so leads to little thermal energy transfer and is not considered destructive.[3,9] It can accelerate bone regeneration and increase cell proliferation, protein synthesis and cytokine production by fibroblasts, osteoblasts and monocytes in culture.[6,9,10] Furthermore, it was also reported that PLIUS does not affect cell viability and can increase glycosaminoglycan (GaG) synthesis by

cells encapsulated in alginate.[8,11,12] However, studies on PLIUS on alginate-encapsulated 3T3 mouse fibroblasts have not been reported.[13,14]

Alginate has been widely used in a variety of biomedical applications, including drug delivery and cell transplantation.[13] It has a number of advantageous features, including low cytotoxicity and non-immunogenicity. An important advantage is that it can be made to form gels simply by adding calcium ions, so there is unlikely to be mechanical damage to suspended cells during gelation.[15] One problem with cell encapsulation in alginate is that it can prevent cell proliferation and decrease metabolic activity,[16] hindering

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matrix formation. However, our previous report shows that the concentration of alginate used for encapsulation has a marked influence on the cell viability and proliferation.[17] These results have been used to choose the alginate concentration and cell seeding density to avoid the adverse effects of culturing in an alginate gel.

In this study, 3T3 mouse fibroblast cells were *cultured* in alginate discs, and the effect of PLIUS on the cell number (measured by Hoechst 33258 assay), collagen synthesis (measured by hydroxyproline assay) and GaG content were evaluated (measured by 1,9-dimethylamine blue (DMB) assay). The spatial distribution of GaG deposition was determined by Alcian blue staining.

Material and methods

Materials

Sodium alginate (characterized by a viscosity of 0.02–0.04 Pa s, 1% solution at 25°C, Cat no: 180947), Dulbecco's modified Eagle's medium (DMEM), chloramine-T, Ehrlich's reagent, perchloric acid, *trans*-4-hydroxy-L-proline, trypsin (0.05% solution in ethylenediaminetetraacetic acid (EDTA)), DMB dye and a DNAQF DNA Quantification Kit (for performing the Hoechst 33258 assay) were purchased from Sigma–Aldrich (Poole, Dorset, UK). Foetal calf serum was purchased from PAA Laboratories (Farnborough, Hampshire, UK).

Encapsulation of fibroblast cells

Mouse fibroblasts (NIH 3T3 murine fibroblast; ATCC, Middlesex, UK) were cultured and treated with trypsin solution in EDTA (0.05%) to detach them from the T-flask and were resuspended in DMEM (5 mL). The cells were counted and suspended at the required density (0.76×10^6 cells/mL) for Alcian blue staining and (2.0×10^6 cells/mL) for other experiments by mixing with 2% (w/v) sodium alginate solution. The cell suspension was poured into 12-well plates containing filter paper and filled with calcium chloride (100 mM)[18] and incubated for 2 h in a 37°C incubator with 5% CO_2. The resulting constructs were transferred to a six-well plate and then washed three times with phosphate-buffered saline (PBS) (100 mM). DMEM (5 mL) was added to each well, and the constructs were incubated at 37°C with 5% CO_2; the medium was changed every 3 days for a period of up to 10 days.

PLIUS exposure

A Sonopuls 491 (Enraf-Nonius, Rotterdam, Amsterdam, the Netherlands) US source was used. Its transducer was immersed in a water bath filled with deionized water maintained at 37°C[6] and treated with a chemical solution (SigmaClean water bath, Sigma-Aldrich Poole, Dorset, UK) to prevent bacterial and fungal growth. The deionized water was changed every week. The six-well plate containing the constructs was placed on top of the transducer; the control group was maintained in the same conditions without being exposed to US. The US stimulation was performed for 5 min every day for 10 days at a frequency of 1 MHz and an intensity of 0.2 W/cm² with a 20% duty cycle (i.e. 20 pulses were emitted in 1 s, so that each pulse had a duration of 0.05 s). The treated and control samples were analysed, at regular intervals, to determine cell number and collagen content. The treatment was conducted up to 10 days as an initial stage to observe if the PLIUS has an effect on fibroblast cells in three dimension culture. Previous studies on fibroblast cells in monolayer culture have been reported and showed positive results with regard to cell proliferation and collagen synthesis.[6] The chosen dose in this study was based on a previous study that used PLIUS to treat bovine intervertebral disc cells cultured in alginate beads and fibroblast in monolayer culture.[6,8]

Assay for fibroblast proliferation

Constructs, which had been cultured for 4, 8 and 10 days, were vacuum dried for 2 days and then digested in papain solution (1 mL; 125 μg/mL).[19] The cell number was determined by a Hoechst assay using a DNAQF DNA Quantitation Kit. Hoechst 33258 dye (200 μL; 2 μg/mL) was added to samples (10 μL) in a 96-well plate. Fluorescence (excited at a wavelength of 360 nm) was measured using a spectrophotometer (Promega glomax; Promega, Southampton, UK) at an emission wavelength of 460 nm, at ambient temperature. The total cell number was determined from a plot of fluorescence intensity against results from known densities (2.0–36.0×10^4 cells/mL) of 3T3 fibroblasts.

Measurement of collagen content

Collagen production was determined using a hydroxyproline assay[20,21] performed on samples cultured up to 10 days. A suspension (500 μL) of the sample that had been digested with papain was hydrolysed with hydrochloric acid (HCl) (12 M) at 110°C for 20 h and was then left in a vacuum incubator overnight to allow excess HCl to evaporate.[22,23] The samples were reconstituted in 50% isopropanol with activated charcoal[20,24] and then filtered through Fisherbrand filter paper (QL100, medium/fast flow rate, Fisherbrand Loughborough, Leicestershire, UK). Chloramine-T solution and *p*-dimethylaminobenzaldehyde solution (Ehrlich's reagent) were sequentially added to each filtered sample. The optical densities of the samples were measured using a spectrophotometer (Cecil CE 1020; Cecil Instrument, Cambridge, UK) at a wavelength of 550 nm. The hydroxyproline contents of the samples were determined from a plot of absorbance against concentration (0–12 μg/mL) for *trans*-4-hydroxy-L-proline.
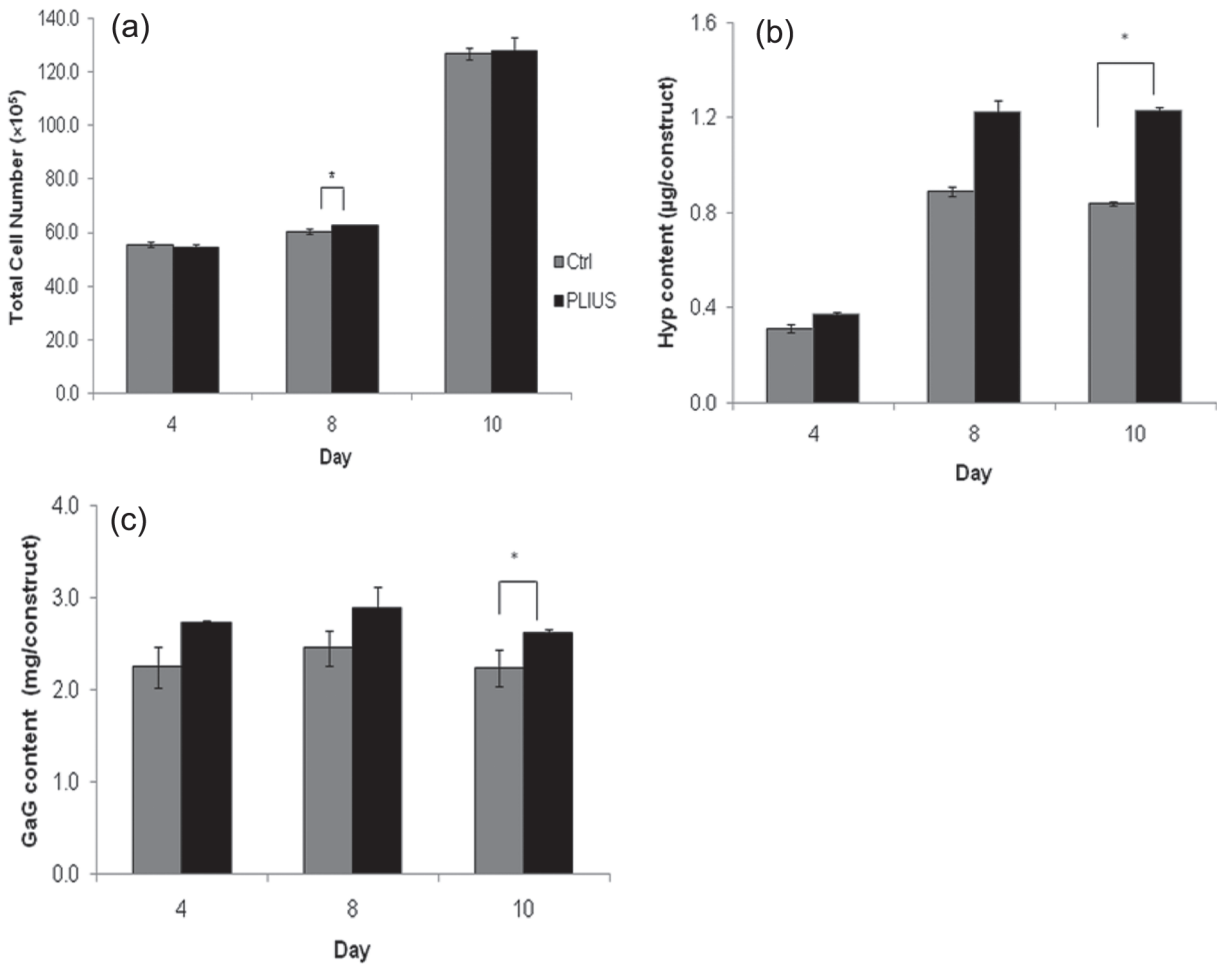
Figure 1. Effect of PLIUS treatment on (a) cell proliferation study, (b) collagen production and (c) GaG production. In (b), collagen is quantified by a hydroxyproline (Hyp) assay. Significant ($p < 0.05$) differences are marked by an asterisk.
PLIUS: pulsed-low intensity ultrasound; GaG: glycosaminoglycan.

Measurement of GaG content

The GaG content was quantified by a previously described method[25] with a slight modification. Samples that have been digested with papain (40 μL) were added to 250 μL DMB dye of pH value 1.5, in order to minimize the reaction of the dye with the alginate.[14] The absorbance was at a wavelength of 600 nm in the dark, to maintain the stable condition of the dye[26] (using a Promega glomax spectrophotometer, Promega). The GaG contents of the samples were determined by comparison of results from whale chondroitin-6-sulphate (0–100 μg/mL).
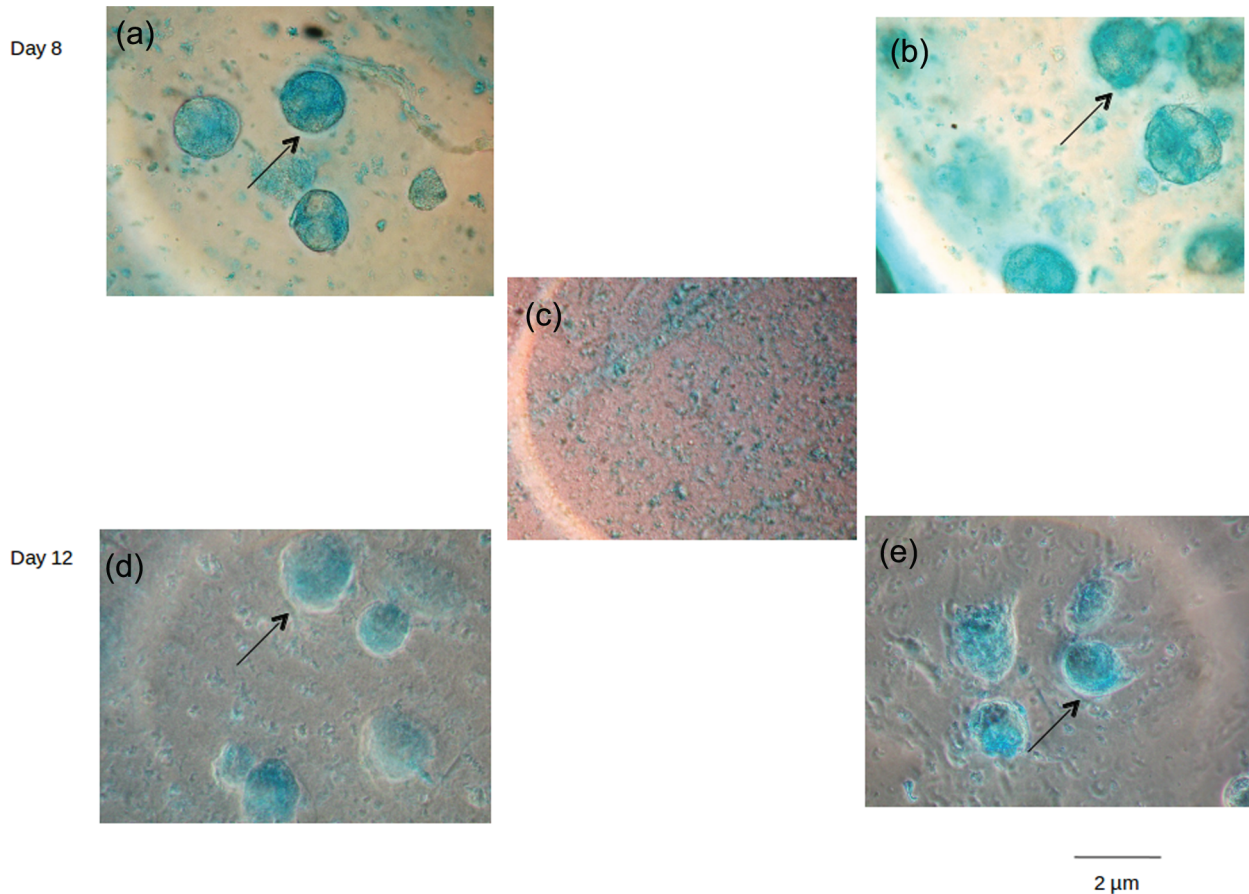
Alcian blue staining

Alcian blue staining for qualitative detection of GaG was performed for up to 12 days. This was done to determine whether there is any difference to the GaG deposition between control and treatment group if the study was prolonged to more than 10 days. The encapsulated cells were fixed with 10% formalin for 20 min and then washed with PBS. The gels were then stained with Alcian blue dye for 48 h (0.05% Alcian blue in 3% acetic acid, pH 1.5 and 0.3 M magnesium chloride ($MgCl_2$)).[27] The alginate/cells were then washed sequentially with 3% acetic acid, 3% acetic acid and 25% ethanol, 3% acetic acid and 50% ethanol and 70% ethanol. The encapsulated cells were observed using a light microscope (Axiolab; Zeiss, Oberkochen, Germany).[27,28]

Statistical analyses

The Shapiro–Wilk test[29] showed that the collagen content results were not normally distributed and were compared using the Mann–Whitney test. The rest of the data were normally distributed data and were compared using an independent *t*-test.[29] Groups were considered to be significantly different for a probability $p < 0.05$. In Figures 1 and 3, differences that correspond to $p < 0.05$ are marked by a single asterisk and differences with $p < 0.01$ are marked with a double asterisk.

Figure 2. Alcian blue staining: (a) control group (Ctrl) after 8 days, (b) treated group (PLIUS) after 8 days, (c) alginate gel only, (d) control group (Ctrl) after 12 days and (e) treated group (PLIUS) after 12 days. The dark blue surrounding the cells represent positive results on detection of GaG.
PLIUS: pulsed-low intensity ultrasound; GaG: glycosaminoglycan.

Results

The total number (cell proliferation study) of cells encapsulated in alginate was determined after 4, 8 and 10 days of PLIUS treatment using the Hoechst 33258 assay (Figure 1(a)). Although at 8 days of the treatment the result appeared to be significant ($p < 0.05$), the difference is very little and is not consistent with results at other times.

Figure 1(b) shows that PLIUS has a significant effect on the production of collagen by the cells. Although there is an increase in collagen production at 4, 8 and 10 days in culture, in both treated and control groups, there is always a significantly higher ($p < 0.05$) collagen production in the treated group.

Figure 1(c) shows a significant ($p < 0.05$) increase in GaG production by cells treated with PLIUS after 10 days in culture. There appears to be an increased GaG production in the treated groups after 4 and 8 days, but this apparent difference is not statistically significant. In both treated and controlled groups, GaG production appears to increase from days 4 to 8 and then decrease.

Alcian blue staining (Figure 2) shows that GaGs are deposited around the cells from days 8 to 12. The results of these experiments were not quantitative, but there is no qualitative difference in the location of the deposited GaG, or the intensity of staining, between the control group and the cells treated with PLIUS.

Discussion

The results presented here suggest that PLIUS has no appreciable effect on fibroblast proliferation (Figure 1(a)). This finding was supported by a previous study that reported that cells encapsulated in alginate did not show any significant increase in the number when treated with PLIUS.[8] However, there are also contradictory results that suggest that PLIUS can increase DNA synthesis (i.e. cell proliferation) in monolayers (human fibroblast and osteoblast) and rabbit intervertebral discs encapsulated cells.[6,12] Nevertheless, there were many factors that may influence these contradictory results. The PLIUS setting, cell type and conditions that were used in the present study were
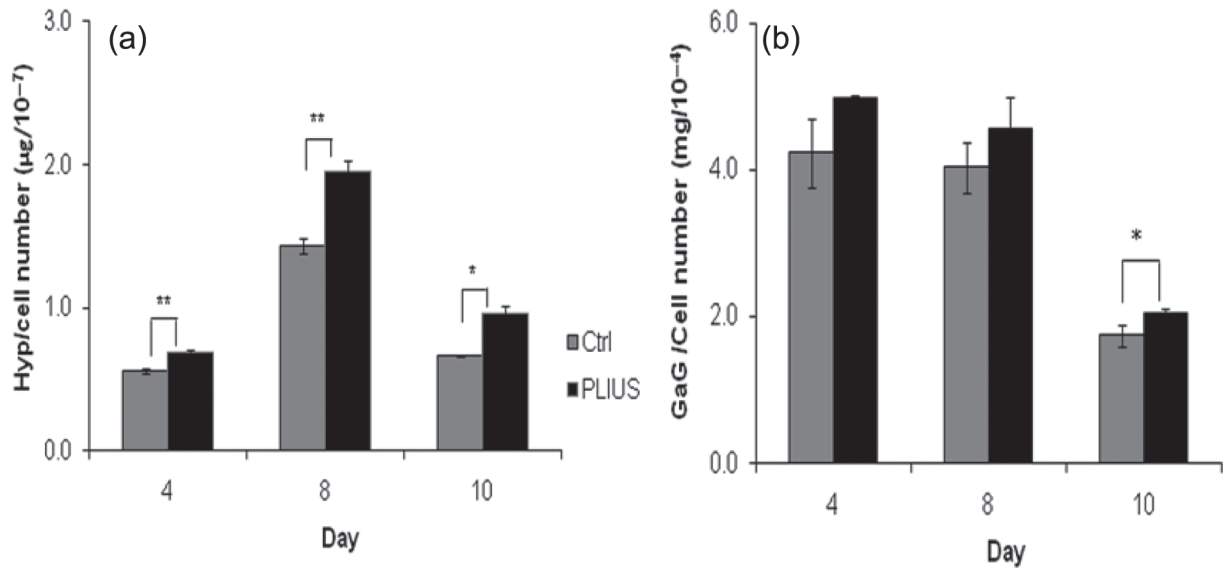
Figure 3. (a) Hydroxyproline (Hyp) content and (b) GaG content divided by cell number for PLIUS treated and control (Ctrl) groups after 4, 8 and 10 days in culture. Significant ($p < 0.05$, $p < 0.01$) differences between the groups are indicated by single and double asterisks, respectively.
PLIUS: pulsed-low intensity ultrasound; GaG: glycosaminoglycan.

totally different from those in published work.[6,12] There is no suggestion, in the work reported here or in any of the articles cited, that treating the cells with PLIUS decreases the number of cells, that is, the cells are not harmed by treatment with US under these conditions.

The ability of PLIUS to induce increased collagen production, in this study, is consistent with the results of other studies. In monolayer culture, rat tenocytes,[30] human fibroblast, osteoblast and monocytes,[6,31] has been reported that collagen production can increase when exposed to PLIUS. Moreover, in cell–scaffold constructs formed from a range of materials, it has also been shown that collagen deposition increases following treatment with PLIUS.[8,11,12,32]

It also appears that PLIUS treatment enhances GaG production. This result is consistent with the work of Miyamoto et al.[8] who reported that PLIUS exposure enhanced collagen and proteoglycan synthesis in bovine intervertebral disc cultured in alginate beads. The decrease in GaG production, in both treated and control groups, after cells had been cultured for 10 days, could be a result of the alginate gel degrading and releasing cells to the culture medium. It is well known that hydrogels such as alginate have the tendency to swell and dissolve in an aqueous environment especially in the presence of calcium chelators, monovalent ions (potassium (K^+), sodium (Na^+), etc.) and non-cross-linking divalent ions.[33]

Figure 3(a) and (b) shows the effect of PLIUS treatment on the production of collagen and GaG per cell. It can be seen that treatment with PLIUS leads to enhanced collagen production by each cell. However, after 8 days, the collagen production per cell decreases for both treated and control

groups. This is because the total number of cells increased at this time, as shown in Figure 1(a). Doan et al.[6] considered this increase in cell number to be a deleterious effect because the cells are involved in cellular division instead of collagen production. Their belief is supported by a previous study showing that fibroblasts submitted to PLIUS (intensity = 0.2–0.6 W/cm^2) at a 10% and 20% duty cycle can maintain their shape and integrity and also increase in number[34]; the intensity and duty cycle for PLIUS used in the present study are within this range. However, it is not clear why the cell number increased after 10 days of PLIUS treatment. With this, there might be another phenomenon that caused the cells to proliferate but did not enhance production of extracellular matrix (ECM). It appears from Figure 3(a) that PLIUS treatment may lead to a small, but significant, increase in GaG production per cell, after 10 days in culture. However, the results at other times are not significant.

Treatment with PLIUS may be beneficial in tissue engineering for inducing fibroblasts to make stronger ECM, perhaps leading to more effective tissue regeneration and repair. Collagen reinforces ECM and, therefore, connective tissues leading to increased stiffness and strength.[35] Therefore, increased collagen production, as a result of PLIUS treatment, is likely to result in stronger tissues. It has been suggested previously that PLIUS or therapeutic US generally could enhance bone repair,[36,37] and repair of intervertebral disc cells[12] and accelerate cartilage formation.[38] Collagen production can also be enhanced by transforming growth factor beta-1 (TGF-β1), insulin and ascorbic acid.[35,39,40] However, physical stimulation of cells

has several advantages over biochemical stimulation; physical stimulation carries no risk of toxicity, has a wide margin of safety and may be used alone or, if necessary, in conjunction with biochemical stimulation.[8]

Funding

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