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Interaction between acoustic intensity and focal depth in low-intensity focused ultrasound analgesia: A 2×2 factorial study in a rat model of inflammatory pain

Fengjun Ni
Shanghai University of Traditional Chinese Medicine
Yong Yao
Shanghai University of Traditional Chinese Medicine
Xiaochen Weng
Shanghai University of Traditional Chinese Medicine
Huayuan Yang
yhy191@sina.cn
Shanghai University of Traditional Chinese Medicine

Research Article

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Abstract

Objective

In traditional acupuncture, the depth of needle insertion and the intensity of stimulation are operator-dependent and lack objective quantification. This study aimed to determine whether focal depth and acoustic intensity affect analgesic efficacy and whether there is an interaction between them.

Methods

We developed a focused low-intensity pulsed ultrasound (FLIPUS) device, named Ultrasound Needle-Free Acupuncture (UNFA), which allows for the independent control of acoustic intensity (0.03–3.0 W/cm²) and focal depth (3 or 5 mm). Using a 2×2 factorial design in a rat model of complete Freund's adjuvant (CFA)-induced inflammatory pain (n = 8 per group), we tested the main effects and interaction of intensity (0.03 vs. 1.0 W/cm²) and depth (3 vs. 5 mm) on mechanical and thermal pain thresholds, paw edema, and open field centre distance.

Results

There was a significant main effect of focal depth on all four outcomes (p = 0.008, 0.038, 0.0037, and 0.0016, respectively), with shallow (3 mm) stimulation consistently outperforming deep (5 mm) stimulation. There was a trend favouring low-intensity stimulation over high-intensity stimulation. No significant interaction between intensity and depth was detected. The optimal parameter set was the US1 combination (shallow depth + low intensity). Furthermore, this significantly reversed the upregulation of key pain-related proteins (PLA2G2A, NTRK1, and BDKRB2) in the spinal cord, providing molecular mechanistic support for the behavioural findings.

Conclusion

Focal depth is the primary determinant of the analgesic efficacy of ultrasound-based acupuncture (UNFA). These findings provide a parametric benchmark for ultrasound-based acupuncture standardisation. Based on the observation that specific parameter combinations (shallow depth and low intensity) produce distinct behavioural and molecular outcomes, future studies could explore whether quantifiable physical parameters of acupoint stimulation can be systematically mapped to subjective sensations, such as *de qi* (needle sensation).

Simple Summary

Acupuncture is widely used for pain relief, but its effectiveness depends heavily on the practitioner's experience, particularly in terms of how deeply the needle is inserted and how strongly it is rotated or

moved. These factors are difficult to standardise. In this study, we developed a new ultrasound-based, needle-free device that can precisely control the depth and strength of stimulation. Using a rat model of inflammatory pain, we discovered that stimulation depth was the most significant factor: shallow stimulation at 3 mm provided more effective pain relief than deep stimulation at 5 mm. The best pain relief was achieved with a combination of shallow depth and low strength. These results provide clear guidance on the use of ultrasound-based acupuncture and open up new possibilities for studying the biological basis of acupuncture sensations using measurable physical parameters.

1. Introduction

Low-intensity therapeutic ultrasound is widely used in preclinical pain research thanks to its non-invasive nature, ability to penetrate deep into tissue, and capacity to deliver high-resolution mechanical stimulation [1–6]. In acupuncture, ultrasound-based, needle-free technologies have recently emerged as a potential alternative to conventional needles. These technologies offer advantages such as reduced infection risk, elimination of needle phobia, and precise, quantifiable parameter control [7–9]. However, several fundamental issues remain unresolved.

Firstly, in traditional acupuncture, the depth of needle insertion and the intensity of manipulation (e.g., twisting or lifting and thrusting) are known to influence therapeutic outcomes. However, these parameters are largely operator-dependent and lack objective quantification. This subjectivity poses a major barrier to standardisation, multicentre trials, and evidence-based evaluation. Secondly, the concept of '*de qi*' (needle sensation), which is described as soreness, numbness, distension, or heaviness around the acupoint, is considered essential for therapeutic efficacy in traditional Chinese medicine [10], yet its physical and physiological correlates remain poorly understood [11, 12], largely because no tool exists to manipulate and measure stimulation parameters precisely in vivo. Thirdly, although ultrasound-based acupuncture devices have been suggested [13], existing systems usually do not permit the independent control of important stimulation parameters, such as acoustic intensity and focal depth. This makes it impossible to systematically investigate the interactions between these parameters. Fourthly, irrespective of whether traditional needle acupuncture or ultrasound-based acupuncture is considered, no study has investigated the potential interaction between stimulation depth and intensity. It is not known whether these two parameters act independently, synergistically, or antagonistically. This knowledge gap can only be addressed by a factorial design capable of estimating both main effects and interactions [14].

To address these gaps, we developed a UNFA system. This device enables the independent digital control of two essential stimulation parameters: acoustic intensity (0.03–3.0 W/cm²) and focal depth (3 or 5 mm). The focal spot diameter (0.4–0.7 mm) is comparable to that of a conventional acupuncture needle (0.3–0.35 mm).

According to the existing literature, the reported values for the intensity of ultrasound acupoint stimulation are predominantly concentrated between 0.03 and 1.0 W/cm², and the depth of needling at

Zusanli (ST36) in rats is typically reported to range from 3 to 5 mm [7, 15–17].

Using this platform, we were able to address questions that have previously been difficult to answer using conventional acupuncture tools, such as whether acoustic intensity and focal depth affect analgesia independently. Do they interact? Most importantly, which parameter, whether intensity or depth, is the primary determinant of efficacy?

To answer these questions, we used a 2×2 factorial design in a rat model of inflammatory pain induced by complete Freund's adjuvant (CFA). This design is particularly suitable for this purpose as it enables the simultaneous estimation of the main effects of two factors (acoustic intensity and focal depth) and their interaction. This is a capability lacking in conventional one-factor-at-a-time designs [18]. The two factors were acoustic intensity (0.03 vs. 1.0 W/cm²) and focal depth (3 vs. 5 mm), yielding four experimental groups (US1–US4). The primary outcome measures were mechanical and thermal pain thresholds, paw edema, and open field centre distance. We hypothesised that focal depth would be a major determinant of analgesic efficacy and that a specific combination of intensity and depth would produce the optimal outcome.

The results demonstrate that focal depth is the primary determinant of UNFA analgesia. Shallow stimulation (3 mm) was significantly more effective than deep stimulation (5 mm) across all four outcome measures. There was a consistent trend favouring low-intensity stimulation over high-intensity stimulation, although the main effect of intensity did not reach statistical significance. Notably, no significant interaction was detected between intensity and depth, indicating that these two parameters act independently in this model. The optimal parameter set was the US1 combination (shallow depth + low intensity).

These findings advance the technical development of ultrasound-based acupuncture and provide a methodological framework for the objective quantification of stimulation parameters, a long-standing challenge in complementary medicine research. Furthermore, they provide the first parametric benchmark for the reproducible, evidence-based application of ultrasound-based acupuncture; they fill the knowledge gap regarding parameter interactions; and they demonstrate the value of parameter-controllable platforms for systematic acupuncture research.

2. Methods

2.1 Animals and ethical statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (approval no. PZSHUTCM2506260005). The study was conducted in accordance with the ARRIVE guidelines and the 3Rs principles (Replacement, Reduction, and Refinement). Thirty-two specific-pathogen-free male Sprague-Dawley rats (200–220 g) were provided by Shanghai Bike Kaiyi Biotechnology Co., Ltd. (Licence No. SCXK (Hu) 2023-0009). The

animals were housed in a controlled environment with free access to food and water, at a temperature of 22 ± 2°C, humidity of 50–65%, and a 12-hour light/dark cycle.

2.2. Inflammatory pain model

After a one-week acclimatisation period, inflammatory pain was induced by injecting 0.1 ml of complete Freund's adjuvant (CFA, BioFroxx, Germany) intraplantarly into the left hind paw under 3% isoflurane anaesthesia. The injection was performed along the midline between the second and third toes. The successful establishment of the model was confirmed 24 hours post-injection by the presence of redness and swelling of the paw, as well as a significant decrease in the pain threshold.

2.3. Experimental design and UNFA intervention

Rats were randomly assigned to four groups of eight using a 2 × 2 factorial design. The two independent factors were acoustic intensity (0.03 vs. 1.0 W/cm²) and focal depth (3 vs. 5 mm). The four groups were defined as follows:

US1: low intensity (0.03 W/cm²) and shallow focal depth (3 mm).

US2: low intensity (0.03 W/cm²) and deep focal depth (5 mm).

US3: high intensity (1.0 W/cm²) and shallow focal depth (3 mm).

US4: high intensity (1.0 W/cm²) and deep focal depth (5 mm).

All interventions were delivered using a custom-built UNFA device (Zanshutech UNFA-A) targeting the bilateral Zusanli acupoint (ST36) (The detailed design and realisation of the custom rat treatment platform can be seen in Supplementary Figure S1). This is located 5 mm below and lateral to the capitulum fibulae on the posterolateral side of the knee joint. The stimulation parameters were set to a fundamental frequency of 0.6 MHz and a duty cycle of 20%. Each 5-minute session was administered once every other day for a total of four sessions (7 days). The primary outcome measures were mechanical and thermal pain thresholds, paw edema, and open field centre distance.

2.4. Measurement of paw edema

Paw thickness was measured using a digital calliper (Mitutoyo, Japan) at baseline (one day before modelling), 24 hours post-CFA (before treatment), and on days 3, 5, and 7 after the first intervention. Each paw was measured three times, and the average was recorded. Paw edema was expressed as the difference in thickness between the left (inflamed) and right (contralateral) paws.

2.5 Pain threshold assessment

All behavioural tests were performed by an observer who was unaware of group allocation.

Thermal pain threshold: The rats were placed individually in acrylic chambers (20 cm × 15 cm × 15 cm) on a glass surface and allowed to acclimatise for 30 minutes. A radiant heat source was then focused on the plantar surface of the left hind paw, and the paw withdrawal latency (PWL) was automatically recorded [19]. A cut-off time of 20 s was used to prevent tissue damage. Each rat was tested three times at five-minute intervals, and the mean PWL was calculated.

Mechanical pain threshold: Rats were placed in transparent acrylic boxes (20 × 20 × 15 cm) on an elevated wire mesh grid. After a 15-minute acclimatisation period, a series of von Frey filaments (0.4–26 g) were perpendicularly applied to the plantar surface of the left hind paw for six to eight s using the up-down method [20]. The 50% paw withdrawal threshold (PWT) was determined as the minimum force eliciting a positive withdrawal response in at least three out of five applications.

2.6. Open Field Test

Spontaneous locomotor activity was assessed using an open field apparatus (110 cm × 110 cm × 170.5 cm). Rats were placed in the centre of the arena, and their behaviour was recorded by an overhead camera for five minutes [21]. The centre distance (the total distance travelled in the central zone) was analysed using EthoVision software (Noldus, the Netherlands). The apparatus was thoroughly cleaned between trials to prevent olfactory cues.

2.7. Western blot analysis (see Supplementary Material)

Spinal cord segments (L4–L6) were harvested and prepared for Western blotting. Detailed procedures are provided in the Supplementary Methods. Antibodies against PLA2G2A, NTRK1, BDKRB2, and β-actin were used. Band intensities were quantified by densitometry and normalised to β-actin.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, USA). A two-way analysis of variance (ANOVA) was used to evaluate the main effects of acoustic intensity (0.03 vs. 1.0 W/cm²) and focal depth (3 vs. 5 mm), as well as their interaction. Tukey's post hoc test was applied for multiple comparisons. The threshold for statistical significance was set at $p < 0.05$.

3. Results

Figure 1 shows the custom-built UNFA device. Figure S2 shows the time course of mechanical and thermal pain thresholds. Figures 2 and Tables 1–2 summarise the effects of acoustic intensity and focal depth on pain-related outcomes at the endpoint (day 7).

3.1. Mechanical pain threshold

A two-way ANOVA revealed a significant main effect of focal depth on the mechanical pain threshold ($p = 0.008$). Pain thresholds were significantly higher for shallow stimulation (3 mm; 18.61 ± 2.54 g) than for

deep stimulation (5 mm; 13.49 ± 2.54 g). However, no significant main effect of intensity (p = 0.356) or intensity × depth interaction (p = 0.452) was observed. Post hoc comparisons showed that the SHALLOW: LOW group (US1, 20.14 ± 2.54 g) had the highest mechanical pain threshold, while the DEEP: HIGH group (US4, 13.34 ± 2.54 g) had the lowest. There was a trend towards significance in the difference between these two groups (mean difference = 6.80 g; 95% confidence interval [CI]: -0.12 to 13.72; p = 0.071) (see Fig. 2A).

3.2. Thermal pain threshold

Similarly, a significant main effect of depth was observed for thermal pain thresholds (p = 0.038). Thermal pain thresholds were significantly higher following shallow stimulation (3 mm; 17.03 ± 2.84 s) than deep stimulation (5 mm; 12.66 ± 2.84 s). The main effect of intensity was not significant (p = 0.151), while the intensity × depth interaction showed a trend towards significance (p = 0.054). Post hoc comparisons revealed that the SHALLOW: LOW group (US1: 20.53 ± 2.84 s) had significantly higher thermal pain thresholds than the DEEP: LOW group (US2: 12.12 ± 2.84 s) (mean difference = 8.41 s; 95% confidence interval (CI): 0.67–16.16; p = 0.029). (see Fig. 2B).

3.3. Paw edema

A two-way ANOVA revealed a highly significant main effect of depth on paw edema (p = 0.0037). Shallow stimulation (3 mm; 1.56 ± 0.60 mm) produced less edema than deep stimulation (5 mm; 2.42 ± 0.60 mm). There was also a trend towards a main effect of intensity (F(1, 28) = 3.319, p = 0.079). Post hoc comparisons showed that the SHALLOW: LOW group (US1, 1.41 ± 0.74 mm) exhibited significantly less edema than the DEEP: HIGH group (US4, 2.78 ± 0.58 mm) (mean difference = -1.37 mm, 95% confidence interval [CI]: -2.42 to -0.31, p = 0.0075) (see Fig. 2C).

3.4. Open field test – centre distance

In the open field test, the main effect of depth on centre distance was highly significant (p = 0.0016). Shallow stimulation (3 mm; 24.44 ± 3.87 cm) resulted in greater centre exploration than deep stimulation (5 mm; 10.89 ± 3.87 cm). No significant main effect of intensity (p = 0.173) or interaction (p = 0.638) was found. Post hoc comparisons revealed that the SHALLOW:0.03 group (US1, mean centre distance: 26.23 ± 3.87 cm) travelled significantly greater centre distances than the DEEP:1.0 group (US4, mean centre distance: 7.28 ± 3.87 cm) (mean difference = 18.95 cm, 95% confidence interval (CI): 4.02 to 33.88, p = 0.009) (see Fig. 2D).

3.5. Summary of findings

A consistent pattern emerged across all four outcome measures (mechanical and thermal pain thresholds, paw edema, and open field exploration): shallow-depth (3 mm) stimulation consistently outperformed deep-depth (5 mm) stimulation. The optimal parameter set was represented by the SHALLOW: LOW (US1) combination. Although the main effect of intensity did not reach statistical significance for most outcome measures (p > 0.05 for mechanical and thermal pain thresholds and p =

0.173 for centre distance), a consistent trend favouring low-intensity (0.03 W/cm²) over high-intensity (1.0 W/cm²) stimulation was observed across all four indicators. This trend reached borderline significance for paw edema (p = 0.079) (see Tables 1 and 2). Supplementary Figure S3 provides representative heatmaps and trajectory maps illustrating the differences in locomotion among groups.

3.6. Western blot analysis of spinal pain-related proteins

To explore the molecular mechanisms underlying the analgesic effects of UNFA, we examined the expression of three pain-related proteins in the spinal dorsal horn of an additional cohort of control, model and US1-treated rats (n = 3–4 per group; see Fig. 3). These proteins were PLA2G2A (a key enzyme that initiates the production of pro-inflammatory lipid mediators), NTRK1 (which encodes the TrkA receptor — the high-affinity receptor for nerve growth factor, and a core component of pain signal transduction) and BDKRB2 (the bradykinin B2 receptor, which mediates the pro-inflammatory and pro-nociceptive effects of bradykinin). The Model group exhibited significantly higher expression levels of all three proteins than the Control group ($p < 0.0001$). Notably, the US1 combination (shallow depth + low intensity) markedly reversed this upregulation, with protein expression levels being significantly lower than in the model group ($p < 0.0001$). These molecular data demonstrate that UNFA, particularly when administered with the optimal US1 parameter set, suppresses spinal pain-related pathways, thereby providing mechanistic support for the behavioural findings.

Table 1
Summary table of all indicators for each group (n = 8)

Group	Mechanical pain threshold (g)	Thermal pain threshold (s)	Paw edema (mm)	Open field center distance (cm)
US1 (3 mm, 0.03 W/cm²)	20.14 ± 2.54	20.53 ± 2.84	1.41 ± 0.74	26.23 ± 5.47
US2 (5 mm, 0.03 W/cm²)	13.62 ± 4.58	12.12 ± 2.84	2.07 ± 0.75	14.51 ± 5.47
US3 (3 mm, 1.0 W/cm²)	17.09 ± 4.10	13.53 ± 2.84	1.70 ± 0.98	22.66 ± 5.47
US4 (5 mm, 1.0 W/cm²)	13.34 ± 4.29	13.19 ± 2.84	2.78 ± 0.58	7.28 ± 5.47

Table 2
Summary of two-way ANOVA Results (n = 8)

Measure	Source of Variation	F-value	P-value	Significance
Mechanical pain threshold	Depth	F(1,28) = 8.156	0.008	**
	Intensity	F(1,28) = 0.88	0.3564	ns
	Interaction	F(1,28) = 0.582	0.452	ns
Thermal pain threshold	Depth	F(1,28) = 4.757	0.0377	*
	Intensity	F(1,28) = 2.178	0.1512	ns
	Interaction	F(1,28) = 4.049	0.0539	ns
Difference in thickness between the left and right feet after intervention	Depth	F(1,28) = 10.05	0.0037	**
	Intensity	F(1,28) = 3.319	0.0792	ns
	Interaction	F(1,28) = 0.5795	0.4529	ns
Distance from the open field center	Depth	F(1,28) = 12.29	0.0016	**
	Intensity	F(1,28) = 1.951	0.1734	ns
	Interaction	F(1,28) = 0.226	0.6382	ns

* $p<0.05$, ** $p<0.01$, ns = not significant.

4. Discussion

4.1 Summary of main findings

This 2×2 factorial study systematically evaluated the independent and interactive effects of acoustic intensity and focal depth on UNFA-mediated analgesia in a rat model of inflammatory pain. The key finding is that focal depth is the primary determinant of analgesic efficacy. Shallow-depth (3 mm) stimulation was found to be consistently more effective than deep-depth (5 mm) stimulation across all four outcome measures: mechanical pain threshold ($p = 0.008$), thermal pain threshold ($p = 0.038$), paw

edema ($p = 0.0037$), and open field centre distance ($p = 0.0016$). While the main effect of intensity did not reach statistical significance, a consistent trend favouring low-intensity (0.03 W/cm²) stimulation over high-intensity (1.0 W/cm²) stimulation was observed across all indicators. The US1 combination (shallow depth + low intensity) consistently represented the optimal parameter set. These findings provide the first experimental evidence that focal depth determines the efficacy of ultrasound-based acupuncture, offering a critical parametric benchmark for its reproducibility.

It also revealed that acoustic intensity and focal depth have an independent influence on UNFA-mediated analgesia, with no significant interaction between the two parameters. While this finding was statistically negative, it suggests that the peripheral mechanisms encoding stimulus intensity and depth are distinct and do not interact within the tested ranges[22, 23]. Intensity is likely dependent on fibre type recruitment or firing rate, whereas depth depends on receptor location (e.g., skin versus deep tissue). The lack of interaction indicates that nociceptive inputs combine additively rather than synergistically or antagonistically. This supports the idea that the spinal cord integrates independent sensory features via parallel pathways[24]. Future studies combining parametric tests with neurophysiological recordings could directly test this hypothesis.

4.2 Why is focal depth the key factor?

The superiority of shallow-depth stimulation can be attributed to the anatomical location of the ST36 acupoint [25]. In rats, the effective depth of the ST36 acupoint has been reported to be approximately 3–4 mm below the skin surface, which closely aligns with our optimal focal depth of 3 mm. Deeper penetration may miss the target tissue, reducing the stimulation of key neural structures (e.g., deep peroneal nerve branches) and diminishing the analgesic effect [26, 27]. Furthermore, ultrasound energy attenuation with tissue depth may also be a factor: higher acoustic intensity is required to achieve comparable mechanical effects at deeper targets, potentially introducing off-target effects [28–30]. Our observation that the US1 (3 mm + 0.03 W/cm²) combination was more effective than the US3 (3 mm + 1.0 W/cm²) combination within the effective depth range suggests that lower intensity may be sufficient and that higher intensity does not provide additional benefit.

Although our findings contradict the conventional belief that deeper acupuncture stimulation produces better analgesic effects, we speculate that this may be related to the anatomical and neuroanatomical stratification of the rat's 'Zusanli (ST36) acupoint region.

Shallow stimulation primarily targets receptors in the skin and subcutaneous tissues that are rich in Aβ fibres (low-threshold mechanoreceptors). The selective activation of Aβ fibres is a well-established mechanism for initiating segmental inhibition via the gate control theory. These fibres mainly convey non-noxious tactile information and terminate in the deep layers of the spinal cord (laminae III–V), where they activate inhibitory interneurons to generate feed-forward inhibition, thereby 'closing the gate' to pain transmission[31, 32].

In contrast, deep stimulation (5 mm) reaches the muscular layer and deep fascia, where it activates a broader population of afferent fibres, including Aδ and C fibres, as well as PROKR2-expressing sensory neurons that specifically innervate deep fascia tissue. Evidence suggests that deep acupuncture at ST36 produces stronger and more sustained analgesic effects due to the activation of a greater number of mechanoreceptors and the involvement of specific neural pathways (e.g., the vagal–adrenal axis). However, in the context of our focused ultrasound stimulation, which differs from traditional needle manipulation, the more diffuse activation of deeper tissues may inadvertently recruit nociceptive inputs that counteract the segmental inhibition initiated by Aβ fibre activation. Therefore, the net analgesic effect depends on the balance between these opposing mechanisms, which may explain why shallow (3 mm) stimulation produced superior outcomes in our study[33, 34].

Importantly, the focal depth of UNFA corresponds directly to the depth of needle insertion in traditional acupuncture, enabling the objective quantification of this previously experience-dependent parameter. The direct equivalence between a physical parameter of the device (millimetres of focal depth) and a clinical parameter of manual acupuncture (millimetres of needle insertion) is a critical advantage of the UNFA platform for translational research.

4.3 Innovation: From Engineering to Methodology

The key strength of this study is its integrated approach. To answer the scientific question of how depth and intensity affect acupoint stimulation, we first developed a dedicated device (UNFA), which is capable of independently controlling these two parameters. Through a 2×2 factorial experiment, we revealed their interaction and identified the optimal parameter combination. Thus, the UNFA device provides a new technological tool for acupuncture research and establishes a parametric benchmark for future studies on ultrasound-based acupuncture parameter harmonisation and preclinical/clinical translation.

To our knowledge, this is the first study to apply a 2×2 factorial design to investigate parameter interactions in ultrasound-based acupuncture. Most previous studies on therapeutic ultrasound for pain management have focused on single-parameter optimisation, without systematically examining potential interactions between parameters [35, 36]. The factorial design employed here enables the simultaneous estimation of main effects and interactions, offering greater statistical efficiency than one-factor-at-a-time approaches.

4.4 The molecular mechanisms underlying UNFA analgesia

Spinal molecular data provide further insight into the analgesic action of UNFA. The optimal parameter set (US1, shallow depth + low intensity) was found to significantly downregulate the expression of PLA2G2A, NTRK1, and BDKRB2 in the spinal dorsal horn. PLA2G2A is a key enzyme in the arachidonic acid cascade that initiates the production of pro-inflammatory lipid mediators, such as prostaglandins. Its suppression likely contributes to reduced peripheral and central sensitisation[37]. NTRK1 encodes the TrkA receptor, which is the high-affinity receptor for nerve growth factor (NGF). NGF is a well-established mediator of inflammatory pain and hyperalgesia[38]. BDKRB2 encodes the bradykinin B2 receptor, which directly sensitises nociceptors and promotes inflammation[39]. The coordinated downregulation of

these three proteins by the US1 parameter set suggests that UNFA may act, at least in part, by inhibiting the spinal amplification of nociceptive signals initiated by peripheral inflammation. This molecular signature is consistent with the behavioural observation that shallow-depth, low-intensity stimulation produces the most consistent analgesic effects. It also supports the conclusion that the optimal parameter combination acts through specific biological pathways rather than non-specific effects.

4.5 Towards the standardisation of acupuncture research

Traditional acupuncture lacks objective quantification of depth and intensity. These parameters are difficult to standardise, quantify, or replicate across different practitioners and institutions, which poses a major challenge for multicentre trials and evidence-based evaluation [40].

In contrast, the UNFA device developed in this study provides a platform that enables independent and precise control of the two key stimulation parameters in a non-invasive manner. This feature enables the systematic investigation of how stimulation intensity and depth affect therapeutic outcomes individually and interactively, as demonstrated by the 2×2 factorial design in the present study.

More broadly, this work provides proof of concept that ultrasound-based, needle-free acupuncture can serve as a standardised, quantifiable, and replicable tool for acupoint stimulation. Such a platform has the potential to facilitate cross-study comparisons and accelerate the translation of acupuncture research into clinical practice by providing a basis for the development of evidence-based parameter guidelines for acupuncture.

This aligns with the broader goal of applying the same standards of objectivity and reproducibility to complementary medicine research as to conventional medical research.

4.6 Opening a new avenue for de qi research

Beyond standardisation, the fact that UNFA technology can be controlled by parameters opens up a new avenue for investigating the biological basis of traditional acupuncture concepts. The physical and physiological correlates of *de qi* are poorly understood, largely due to a lack of tools that can manipulate and measure stimulation parameters precisely in vivo.

With its ability to independently control stimulation depth, intensity, frequency, and duty cycle, the UNFA platform offers a unique opportunity to explore how specific physical parameters of acupoint stimulation translate into distinct biological responses. For instance, does low-intensity, shallow-depth ultrasound preferentially activate cutaneous mechanoreceptors or Aβ fibres? Does deeper penetration engage different neural circuits? Can the sensation of *de qi* be correlated with specific patterns of ultrasound-induced mechanical stress?

Additionally, unlike human subjects, animals cannot verbally report the subjective sensations associated with *de qi*. However, we hypothesise that the sensation of *de qi* in humans may correspond to the activation of specific neural pathways, which could be triggered by defined sets of physical parameters. One such combination is the low-intensity, shallow-depth ultrasound identified in this study. The robust

analgesic and behavioural responses observed in CFA rats following this specific parameter set (US1) could therefore serve as objective surrogate markers for a '*de qi*-like' state in animals. This framework opens a tangible pathway for the first time to investigate the neurobiological underpinnings of *de qi* and its relationship to therapeutic efficacy, effectively translating a traditionally subjective concept into a quantifiable and testable research question.

Future studies integrating USFA with real-time neuroimaging (e.g., fMRI) or electrophysiological recording could help to decipher the neural encoding of *de qi* and its relationship to analgesia. Such interdisciplinary efforts could ultimately bridge the gap between traditional acupuncture theory and modern neuroscience, providing a mechanistic framework to explain why specific stimulation parameters yield specific therapeutic effects.

4.7 Translational implications: from bench to clinic

4.7.1 Clinical translation

As discussed in Section 4.2, the focal depth of UNFA corresponds directly to the depth of needle insertion in traditional acupuncture. This allows the previously experience-dependent parameter to be quantified objectively. However, the relationship between acoustic and manual stimulation intensities requires further investigation. The 'intensity' of manual acupuncture is a composite variable comprising mechanical force, twisting amplitude, lifting-thrusting frequency, and operator technique [41, 42], whereas acoustic intensity is a single physical parameter. Nevertheless, our finding that lower intensity (0.03 W/cm²) produced consistently better outcomes suggests that 'gentle' stimulation may be preferable in the context of inflammatory pain, a hypothesis that can be tested in future comparative studies.

It is important to note that, since both UNFA and manual acupuncture primarily rely on the mechanical stimulation of acupoints, it is theoretically possible to establish equivalence between specific combinations of ultrasound parameters (depth, intensity, frequency, and duty cycle) and specific manual manipulation techniques (twisting, lifting, and thrusting, and force). Future studies that systematically map these parameter spaces, ideally using dose-response designs and neurophysiological readouts, could bridge the gap between a quantifiable physical stimulus and the traditionally experience-dependent art of acupuncture manipulation.

4.7.2 Implications for the standardisation and training of acupuncture

The UNFA platform also has direct implications for acupuncture standardisation and training. For instance, in veterinary practice, the depth of needle insertion and the intensity of stimulation are largely dependent on the operator and vary across species and body sizes [43]. UNFA offers a quantifiable and reproducible alternative; for instance, a standardised 'shallow depth + low intensity' protocol could be

developed to treat inflammatory pain in small animals. This parameter-controllable platform thus opens up a new avenue for evidence-based veterinary acupuncture.

4.7.3 Animal welfare and the 3R principles

From an animal welfare perspective, UNFA is non-invasive, eliminating the risk of pain, bleeding, and infection associated with needles, and reducing stress induced by handling. This approach aligns with the 3Rs (Replacement, Reduction, Refinement) and represents an ethical improvement over conventional needle-based acupuncture in animal research [44].

4.8 Limitations and future directions

Several limitations should be acknowledged. Firstly, the small sample size (n = 8 per group) may have limited the study's statistical power to detect small effect sizes, particularly about intensity. However, the consistent trend favouring low intensity across all four measures suggests that a larger sample size would be required to reveal significant intensity effects.

Secondly, for the sake of simplicity, the present study did not include a group receiving traditional needle acupuncture, a group receiving a placebo (e.g., inactive ultrasound), or a group left to recover naturally. However, the primary objective was to test for interactions between two specific physical parameters (acoustic intensity and focal depth), which was achieved using a 2×2 factorial design. Furthermore, our spinal molecular data (Fig. 3 and Figure S4) provide compelling evidence: the expression levels of key pain-related proteins (PLA2G2A, NTRK1, and BDKRB2) were significantly higher in the Model group than in the Control group, and this increase was robustly reversed in the US1 group (shallow depth and low intensity). These molecular changes confirm the validity of the CFA model directly and demonstrate the specific therapeutic action of UNFA. This compensates for the absence of behavioural controls at a mechanistic level.

Thirdly, the molecular mechanisms underlying depth-dependent analgesia were not investigated directly. Our complementary Western blot data (see Supplementary Material) suggest that UNFA may suppress inflammatory and pain-related pathways, but dedicated mechanistic studies are required. Fourthly, the present study focused on short-term outcomes (7 days), so the long-term analgesic effects of UNFA remain unknown. Fifth, focal depth adjustment was limited to two levels (3 and 5 mm) due to hardware constraints of the device. Continuous depth adjustment would enable more precise parametric optimisation. Sixthly, the relationship between intensity and response requires further investigation across a wider range of intensities. Seventhly, the relationship between acoustic intensity and manual stimulation intensity — a composite variable involving force, frequency, and technique — remains to be established. Direct equivalence between the two cannot be assumed from the current data.

Future research should explore the optimal depth–intensity combination in other pain models (e.g., neuropathic and postoperative pain), investigate long-term safety and efficacy, and further develop the technical capabilities of the UNFA platform, particularly about continuous depth adjustment and real-time feedback control. Direct comparative studies between UNFA parameters and manual acupuncture

manipulations are also needed to establish whether acoustic intensity corresponds to traditional concepts of stimulation 'strength'.

5. Conclusions

This 2×2 factorial study demonstrates that focal depth is the primary determinant of UNFA analgesic efficacy. Shallow (3 mm) stimulation significantly outperformed deep (5 mm) stimulation across multiple pain-related outcomes. There was a consistent trend favouring low intensity (0.03 W/cm²) over high intensity (1.0 W/cm²) stimulation, and the US1 combination (shallow depth + low intensity) represents the optimal parameter set. The key strength of this study lies in its integrated approach. We developed a device capable of independently controlling stimulation depth and intensity and used it to demonstrate that focal depth is the primary determinant of analgesic efficacy. Based on this finding, we propose that platforms with controllable parameters may be a useful technological means for future research aimed at standardising acupuncture parameters and investigating the relationship between physical stimulation and traditional concepts such as de qi.

Declarations

Ethics, Consent to Participate, and Consent to Publish declarations

As this study does not involve human participants, data or tissue, consent to participate and consent for publication are not applicable. All animal procedures were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (approval no. PZSHUTCM2506260005, granted 26 June 2025). The study was conducted in accordance with the ARRIVE guidelines and the 3Rs principles.

Availability of data and materials

The original data presented in this study can be found in the article itself and in the supplementary materials. Further enquiries can be directed to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors‘contributions

F.N. and Y.Y. conceived and designed the study. F.N. developed the methodology, performed the software analysis, conducted the formal analysis and investigation, curated the data, prepared the original draft, and created the visualizations. Y.Y. and X.W. contributed to the validation process. Y.Y., X.W., and H.Y. reviewed and edited the manuscript. H.Y. supervised the project, provided resources, and acquired funding alongside X.W. All authors read and approved the final manuscript.

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Clinical trial number

Not applicable.

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Figures

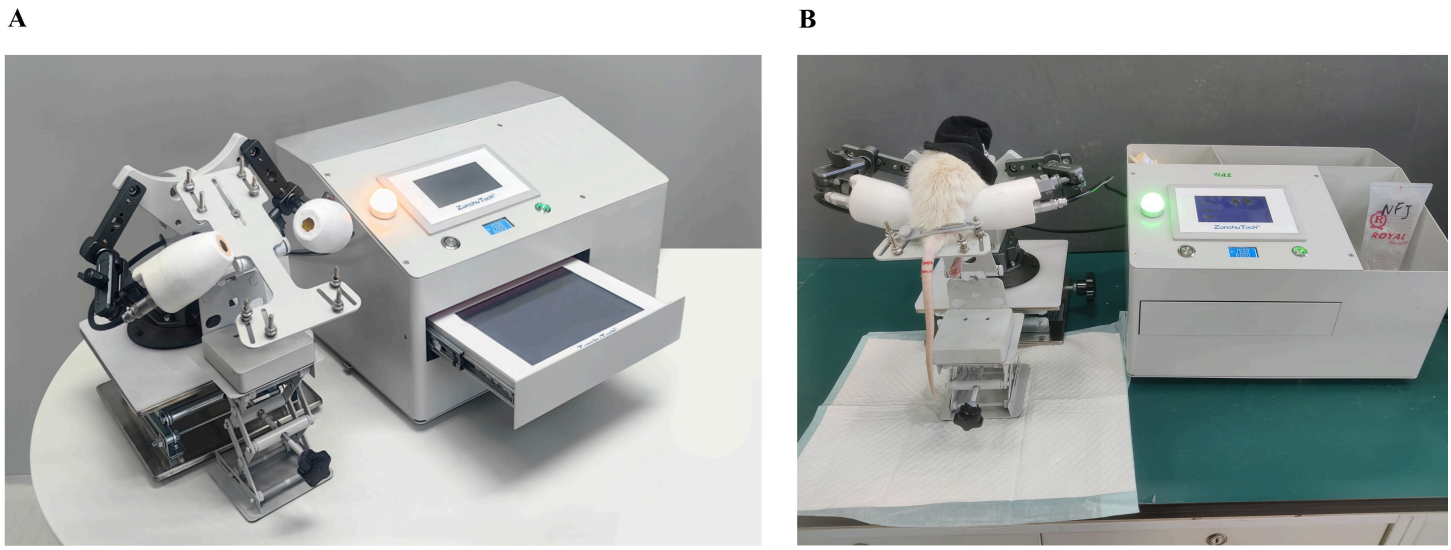


Figure 1

The Zanshutech UNFA-A device and its application in a rat model.
(A) Photograph of the UNFA-A device, showing the main control unit and the dual-transducer positioning arm.
(B) Representative image of UNFA treatment applied to the bilateral Zusanli (ST36) acupoints. Rats were lightly anesthetized with isoflurane and secured on a custom-made platform. A black cloth was placed over the head to minimize stress during the procedure.

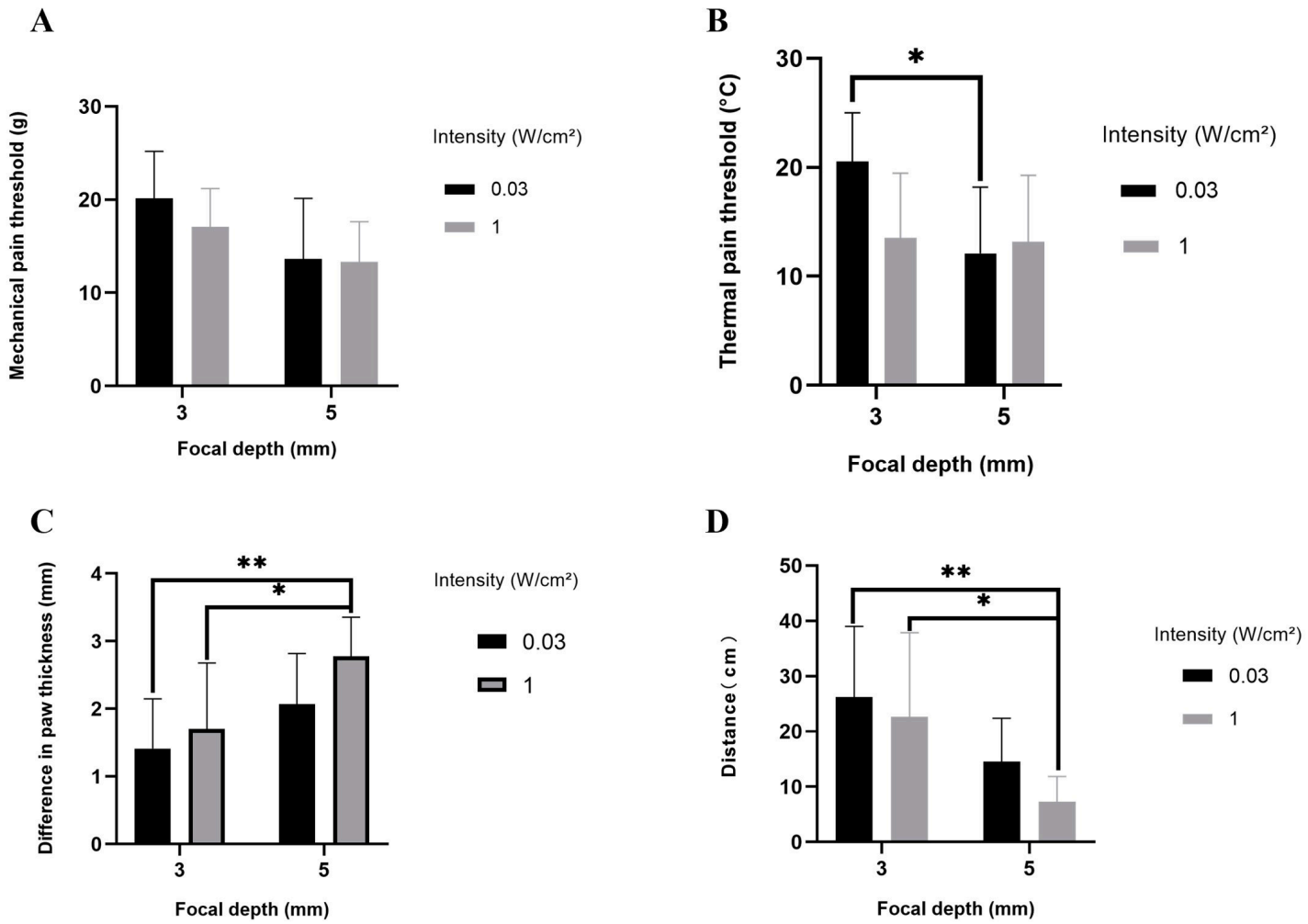


Figure 2

Effects of focal depth and acoustic intensity on pain-related outcomes.

Pain-related outcomes in CFA rats (n = 8 per group) were measured on day 7. (A) Mechanical pain threshold. Data are presented as mean ± SD. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. A significant main effect of focal depth was observed ($p = 0.008$), but no significant differences were found between individual groups in post hoc comparisons.

(B) Thermal pain threshold. Data are presented as mean ± SD. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test.*$p < 0.05$, **$p < 0.01$ compared with the DEEP:HIGH (US4) group.

(C) Paw edema (difference in paw thickness between left and right feet). Data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$ compared with the DEEP:HIGH (US4) group.

(D) Distance traveled in the open field center. Data are expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$

compared with the 5 mm depth / 1 W/cm² group.

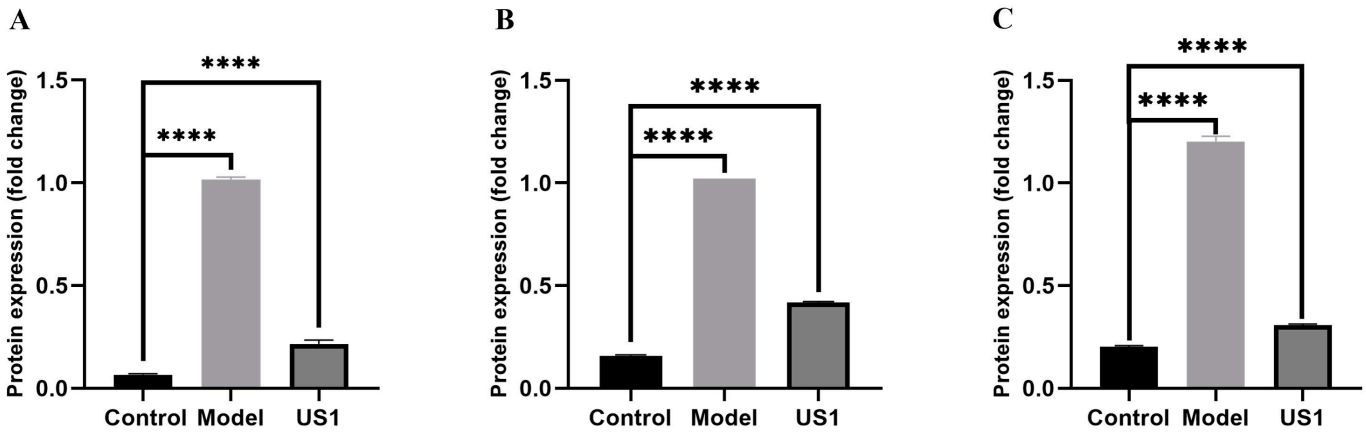


Figure 3

Quantitative analysis of pain-related protein expression in the spinal dorsal horn.
(A) PLA2G2A, (B) NTRK1, and (C) BDKRB2 protein expression normalized to β-actin. Data are presented as mean ± SD (Control, n=4; Model, n=3; US1, n=4). Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test. ****p < 0.0001.

Abbreviations: CFA, complete Freund's adjuvant; SD, standard deviation; US1, 3 mm + 0.03 W/cm²; US2, 5 mm + 0.03 W/cm²; US3, 3 mm + 1.0 W/cm²; US4, 5 mm + 1.0 W/cm².

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